

PATENT SPECIFICATION

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(54) ANTIBIOTIC A-35512 AND PROCESS FOR PRODUCTION THEREOF /

(71) We, ELI LILLY AND COMPANY, a corporation of the State of Indiana, United States of America, having a principal place of business at 307 East McCarty Street, City of Indianapolis, State of Indiana, United States of America, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

Antibiotic A-35512 mixture comprising microbiologically active, related factors A, B, C, E, F, G and H, is produced by submerged aerobic fermentation of *Streptomyces candidus* NRRL 8156. The individual A-35512 factors are separated and isolated by chromatography. A mild acid hydrolysis of A-35512 factor B removes several sugars to form A-35512 factor B aglycone. The A-35512 antibiotics are antibacterial and growth-promoting agents and increase feed-utilization efficiency in ruminants and poultry. In addition, A-35512 factor B is useful in the treatment of dental caries and acne.

The A-35512 antibiotics are closely related, glycopeptide antibiotics. Antibiotic A-35512 factor B, the most completely characterized member of the A-35512 antibiotic complex, appears to be a new member of the group of peptide-containing antibiotics which includes vancomycin (U.S. Patent No. 3,067,099), A-4696 A, B and C (U.S. patent No. 3,952,095), evoparcin (U.S. Patent No. 3,855,410), ristomycin A (Lomakina, N., 7th International Symposium of Chemistry of National Products, Riga, Latvia page 625, 1970) and ristocetin A (U.S. Patent No. 2,990,329). The A-35512 antibiotics differ from these known antibiotics in, for example, movement in various chromatographic systems and amino-acid and sugar content.

Although many antibacterial agents are known today, the need for new, improved antibiotics continues. One problem in current antibiotic therapy is the fact that antibiotics differ in their effectiveness against pathogenic organisms. Another problem is the fact that organism strains continually develop which are resistant to currently-used antibiotics. Yet another problem is the fact that individual patients often suffer serious reactions to specific antibiotics, due to hypersensitivity and/or to toxic effects. Because of these problems in current therapy one object of this invention is to provide new antibiotics for use against disease caused by microorganisms.

Another object of this invention is to provide additional tools to increase the feed efficiency of ruminant animals and poultry. As the need for food increases feed efficiency becomes of greater importance. Diethylstilbestrol and other estrogens have increased feed efficiency in a number of animals and poultry. There has been concern about the hazard of consuming residues of these feed additives which remain in the meat at the time of consumption. There is a very real economic need for new ways of increasing the efficiency of the limited supplies of feed available in the production of meat of ruminant animals and poultry. Antibiotic A-35512 mixture, its factors and their derivatives are steps forward in that direction.

This invention provides an antibiotic A-35512 mixture comprising factors A, B, C, E, F, G and H; A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G and A-35512 factor H; and the aglycone derivative of factor B, as herein defined.

This invention also provides a process of producing said antibiotic A-35512 mixture comprising factors A, B, C, E, F, G and H; A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G and A-35512 factor H; and the aglycone derivative of factor B comprising:

a) cultivating *Streptomyces candidus* NRRL 8156 or an A-35512 mixture producing mutant thereof in a culture medium containing assimilable sources of carbohydrate, nitrogen, and inorganic salts under submerged aerobic fermentation conditions;

b) optionally, separating antibiotic A-35512 mixture from the culture medium;

c) optionally, isolated antibiotic A-35512 factors A, B, C, E, F, G and H from the antibiotic A-35512 mixture; and

d) optionally, preparing antibiotic A-35512 factor B aglycone from antibiotic A-35512 factor B.

This invention also provides a feed composition suitable for the nutrition of ruminant animals having a developed rumen function comprising a feed and propionate-increasing concentration of a member selected from antibiotic A-35512 mixture, A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor B, A-35512 factor H and A-35512 factor B aglycone, as herein defined and the pharmaceutically-acceptable acid addition salts of A-35512 factors A, B, C, E, F, G and H, and of A-35512 factor B aglycone.

This invention also provides a feed composition suitable for the nutrition of poultry comprising a feed and a feed-efficiency increasing concentration of a member selected from said antibiotic A-35512 mixture, A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G, A-35512 factor H and A-35512 factor B aglycone and the pharmaceutically-acceptable acid addition salts of A-35512 factors A, B, C, E, F, G, and H and of A-35512 factor B aglycone.

This invention also provides a method of increasing feed-utilization efficiency in ruminant animals having a developed rumen function comprising orally administering to said animals propionate-increasing amount of a member selected from said antibiotic A-35512 mixture, A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G, A-35512 factor H and A-35512 factor B aglycone and the pharmaceutically-acceptable acid addition salts of A-35512 factors A, B, C, E, F, G and H and A-35512 factor B aglycone.

This invention also provides a method of increasing feed-utilization efficiency in poultry comprising orally administering to said animals a member selected from said antibiotic A-35512 mixture, A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G, A-35512 factor H and A-35512 factor B aglycone and the pharmaceutically-acceptable acid addition salts of A-35512 factors A, B, C, E, F, G, and H and A-35512 factor B aglycone.

The number and ratio of individual factors co-produced in this antibiotic mixture will vary, depending upon the fermentation conditions used. The antibiotic substances of this invention are arbitrarily designated herein as A-35512 antibiotics. Factor B is the major factor in the A-35512 mixture. The A-35512 factors occur in the A-35512 mixture and are recovered individually from the mixture as hydrochloride salts. Each individual factor may be converted to its free base (ionic chlorine free) form by, for example, chromatography over a weakly-basic ion-exchange resin. In addition to the free base and hydrochloride forms, other pharmaceutically-acceptable salts of the A-35512 factors are also useful. In discussions of utility, the term "A-35512 compound" will be used, for the sake of brevity, to denote a compound selected from the group consisting of A-35512 factors A, B, C, E and H and the pharmaceutically-acceptable salts thereof.

Infrared absorption spectra (in KBr pellet) of the following A-35512 factors are presented in the drawings as follows:

Figure 1 — A-35512 factor A dihydrochloride

Figure 2 — A-35512 factor B dihydrochloride

Figure 3 — A-35512 factor H hydrochloride

Figure 4 — A-35512 factor C dihydrochloride

Figure 5 — A-35512 factor E hydrochloride

Infrared absorption spectrum of A-35512 factor B aglycone (in Nujol mull) is presented in Figure 6.

The A-35512 factors of this invention are closely related compounds. As many as seven antibiotic factors are recovered from the fermentation as the antibiotic A-35512 mixture. The individual factors are separated from each other; and factors A, B, C, E and H are isolated as individual compounds as hereinafter described. The A-35512 mixture is soluble in water; is partially soluble in methanol and ethanol; but is insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane.

A-35512 factor A is a white, amorphous, basic compound. A-35512 factor A has the following approximate elemental composition:

Carbon, 54.29%; hydrogen, 5.19%;
nitrogen, 5.58%; oxygen, 33.76%;
chlorine, 1.69%.

A-35512 factor A dihydrochloride is a white, amorphous, hygroscopic compound. A-35512 factor A dihydrochloride has the following average elemental composition:

Carbon, 51.03%; hydrogen, 5.10%;
nitrogen, 4.75%; oxygen, 34.20%;
chlorine, 4.80%.

The infrared absorption spectrum of A-35512 factor A dihydrochloride in KBr pellet is shown in Figure 1 of the accompanying drawings. The most significant absorption maxima occur at the following frequencies (cm^{-1}): 3405 (strong), 3300 (shoulder), 2950 (weak), 1750 (weak), 1670 (strong), 1625 (shoulder), 1602 (strong), 1520 (strong), 1470 (weak), 1440 (weak), 1405 (weak), 1345 (shoulder), 1312 (medium), 1225 (medium), 1180 (weak), 1130 (weak), 1080 (strong), and 1020 (shoulder).

The ultraviolet (uv) absorption spectra of A-35512 factor A dihydrochloride show, in acidic and neutral methanol, an absorption maximum at 282 nm (ϵ 11,700) and, in basic methanol, an absorption maximum at 292 nm (ϵ 14,000), calculated using a molecular weight of 2,000. The uv spectra of A-35512 factor A dihydrochloride also show end absorption at 225 nm.

A ^{13}C nuclear magnetic resonance spectrum of A-35512 factor A dihydrochloride in D_2O has the following characteristics:

No.	PPM	Height
3	175.3	0.8
4	173.0	2.1
5	172.1	2.0
6	171.4	1.5
7	170.9	2.7
8	170.5	2.3
9	169.5	1.6
10	159.0	1.3
11	157.9	2.3
12	156.2	2.6
13	155.6	2.3
14	155.3	2.4
15	155.4	1.1
16	136.3	1.4
17	136.0	1.0
18	135.1	1.4
19	133.5	1.2
20	129.6	1.6
21	129.1	1.7
22	128.7	1.8
23	127.5	1.0
24	126.0	1.4
25	124.3	2.8
26	122.1	1.6
27	109.9	1.3
28	107.4	1.6
29	101.7	0.9

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	No.	PPM	Height	
	30	77.6	3.8	
	31	76.3	4.6	
5	32	75.5	2.6	5
	33	74.8	2.5	
	34	74.5	2.4	
	35	73.4	3.7	
	36	72.8	6.0	
10	37	72.0	4.4	10
	38	70.9	7.0	
	39	69.6	2.8	
	40	67.4	90.9*	
	41	65.4	1.7	
	42	61.7	3.1	
15	43	56.7	1.7	15
	44	55.5	1.3	
	45	54.7	0.8	
	46	24.6	0.9	
20	47	19.1	1.7	20
	48	17.9	2.0	
	49	17.2	1.9	
	50	16.7	3.2	
	51	16.2	3.0	

* dioxane standard

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A-35512 factor A dihydrochloride has the following specific rotations:

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 $[\alpha]_D^{25} - 100^\circ$ (c 1, H₂O) $[\alpha]_{365}^{25} - 400^\circ$ (c 1, H₂O).

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Electrometric titration of A-35512 factor A dihydrochloride in 66 vol. percent aqueous dimethylformamide indicates the presence of four titratable groups with pK_a values of approximately 7.35, 9.09, 10.49, and 12.44 (initial pH 6.2).

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The molecular weight of A-35512 factor A dihydrochloride, as determined by titration, is about 2106.

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A-35512 factor A dihydrochloride is soluble in water, is partially soluble in methanol and ethanol, but is insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane.

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A-35512 factor A dihydrochloride is stable for 72 hours in aqueous solutions having a pH of from 3 to 10.

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Amino acid analysis of acid-hydrolyzed A-35512 factor A dihydrochloride indicates that A-35512 factor A contains at least five amino-acid residues, one of which is glycine.

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A-35512 factor B is a white, amorphous, basic compound. The approximate empirical formula for A-35512 factor B is C₉₇₋₉₈H₁₀₁₋₁₀₅N₈₋₉O₄₆₋₄₈Cl. A-35512 factor B has the following average elemental composition:

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Carbon, 53.97%; hydrogen, 4.75%;
nitrogen, 5.25%; oxygen, 34.29%;
chlorine, 1.59%.

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This elemental composition is in particular agreement with a preferred empirical formula of C₉₈H₁₀₄N₈O₄₇Cl (Calcd: C, 53.60; H, 4.75; N, 5.74; O, 34.30; Cl, 1.61). An alternative preferred empirical formula is C₉₈H₁₀₃N₈O₄₇Cl (Calcd: C, 54.00; H, 4.75; N, 5.15; O, 34.50; Cl, 1.60).

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The ultraviolet absorption (uv) spectra of A-35512 factor B show, in acidic and neutral methanol, an absorption maximum at 282 nm (ϵ 15000) and, in basic methanol, an absorption maximum at 292 nm (ϵ 16000), calculated using a molecular weight of 2000. The uv spectra of A-35512 factor B also show end-absorption at 225 nm.

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A-35512 factor B has the following specific rotations:

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 $[\alpha]_D^{25} - 123^\circ$ (c 1, H₂O) $[\alpha]_{365}^{25} - 446^\circ$ (c 1, H₂O).

Electrometric titration of A-35512 factor B in 66 vol. percent aqueous dimethylformamide indicates the presence of four titratable groups with pK_a values of approximately 7.15, 8.81, 10.20, 12.00 and the possible presence of another group with a pK_a value greater than 13.50.

The molecular weight of A-35512 factor B, as determined by titration, is about 2143.

A-35512 factor B dihydrochloride is a white crystalline compound (from 50 vol. percent aqueous methanol). Although A-35512 factor B dihydrochloride is hygroscopic and does not exhibit a distinct melting point, a thermogram showed weight loss beginning at 25°C., resulting in a 7.4 percent loss at 121°C.; at 135°C. another loss occurred, resulting in decomposition.

A-35512 factor B dihydrochloride has the following approximate elemental composition (average):

Carbon, 52.57%; hydrogen, 4.80%;
nitrogen, 5.66%; oxygen, 32.86%;
chlorine, 4.51%.

This elemental composition is in particular agreement with another alternative empirical formula of $C_{98}H_{103}N_9O_{47}Cl \cdot 2HCl$ (Calcd: C, 51.93; H, 4.65; N, 5.57; O, 33.20; Cl, 4.65).

The uv spectra of A-35512 factor B dihydrochloride show, in acidic and neutral methanol, an absorption maximum at 282 nm (ϵ 12,000) and, in basic methanol, an absorption maximum at 292 nm (ϵ 14,000), calculated using a molecular weight of 2000. The uv spectra of A-35512 factor B dihydrochloride also show end-absorption at 225 nm.

A-35512 factor B dihydrochloride has the following specific rotations:

$[\alpha]_D^{25} - 128^\circ$ (c 1, H_2O)

$[\alpha]_{365}^{25} - 475^\circ$ (c 1, H_2O).

Electrometric titration of A-35512 factor B dihydrochloride in 66 vol. percent aqueous dimethylformamide indicates the presence of four titratable groups with pK_a values of approximately 7.15, 8.87, 10.30, and 12.10 and the possible presence of another group with a pK_a greater than 13.1.

The molecular weight of A-35512 factor B dihydrochloride, as determined by titration, is about 2027.

A ^{13}C nuclear magnetic resonance spectrum of A-35512 factor B dihydrochloride in D_2O has the following characteristics:

No.	PPM	Height
2	173.0	4.1
3	171.9	3.7
4	171.6	3.3
5	171.0	5.8
6	170.8	5.0
7	169.6	3.6
8	159.0	4.1
9	157.9	4.4
10	157.5	3.7
11	156.6	4.8
12	155.6	6.1
13	155.3	4.2
14	154.9	3.3
15	154.3	4.2
16	151.7	3.3
17	144.3	3.1
18	136.7	3.5
19	136.2	4.9
20	135.4	4.0
21	135.2	4.4
22	133.6	4.2
23	133.3	4.1
24	129.8	1.7

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	No.	PPM	Height	
	25	129.3	3.0	
	26	128.8	2.6	
5	27	127.6	1.5	
	28	126.1	3.9	5
	29	124.2	5.6	
	30	122.4	1.4	
	31	122.0	4.4	
10	32	120.7	3.3	
	33	116.5	2.7	10
	34	109.5	0.8	
	35	108.2	1.1	
	36	107.7	2.7	
	37	104.5	1.7	
15	38	101.8	2.9	
	39	100.9	1.6	15
	40	98.2	1.0	
	41	76.9	1.2	
	42	76.1	1.8	
20	43	74.1	2.0	
	44	73.5	2.7	20
	45	72.7	2.4	
	46	72.3	4.0	
	47	71.0	7.1	
25	48	70.3	2.5	
	49	69.7	2.5	25
	50	67.4	74.7*	
	51	64.6	1.2	
	52	62.0	1.5	
30	53	58.0	1.3	
	54	56.8	1.7	30
	55	55.4	3.9	
	56	54.3	2.5	
	57	24.5	2.0	
35	58	17.9	3.0	
	59	17.2	2.0	35
	60	16.3	2.5	

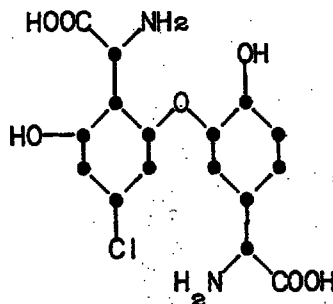
* dioxane standard

40 A-35512 factor B dihydrochloride, crystallized from methanol-water, has the following characteristic X-ray powder diffraction pattern (Cu^{++} radiation, 1.5405 Å, nickel filter, d=interplanar spacing in angstroms): 40

	d	Relative Intensity	
	17.15	100	
45	12.90	80	
	10.85	70	45
	9.25	70	
	8.87	60	
	8.22	50	
50	7.86	50	
	6.93	40	50
	6.20	40	
	5.62	40	
	5.04	05	
55	4.02	02	
	3.54	02	55

60 The infrared absorption spectrum of A-35512 factor B dihydrochloride in KBr pellet is shown in Figure 2 of the accompanying drawings. The most significant absorption maxima occur at the following frequencies (cm^{-1}): 3420 (strong), 3300 (shoulder), 2950 (weak), 1752 (weak), 1675 (strong), 1630 (shoulder), 1605 (strong), 1520 (strong), 1470 (weak), 1440 (weak), 1410 (weak), 1345 (shoulder), 1312 (medium), 1225 (medium), 1180 (weak), 1135 (weak), 1080 (strong), and 1020 (weak). 60

Amino acid analysis of acid-hydrolyzed A-35512 factor B dihydrochloride indicates that A-35512 factor B contains at least five amino-acid residues, one of which is glycine. The four remaining amino-acid residues in A-35512 factor B are complex and appear to be identical to those found in A-35512 factor A. The structure of one of these amino-acid residues appears to be:



Analyses of its acid-hydrolysis products indicate that A-35512 factor B dihydrochloride contains the following sugars: glucose, fucose, mannose, rhamnose, and 3 - amino - 2,3,6 - trideoxy - 3 - C - methyl - L - xylo - hexopyranose. Mild acid hydrolysis of A-35512 factor B dihydrochloride removes the glucose, fucose, mannose, and rhamnose groups to give a characteristic aglycone derivative.

A-35512 factor B dihydrochloride has at least one hydroxyl group capable of esterification.

A-35512 factor B dihydrochloride is soluble in water, is partially soluble in methanol and ethanol, and is insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane.

A-35512 factor B dihydrochloride is stable for up to 72 hours in aqueous solutions having a pH of from 3 to 10.

A-35512 factor C is a white, amorphous, basic compound.

A-35512 factor C has the following approximate elemental composition:

Carbon, 53.93%; hydrogen, 5.15%;
nitrogen, 5.80%; oxygen, 32.35%;
chlorine, 1.90%.

This elemental composition is in agreement with a possible empirical formula of $C_{84}H_{99}N_8O_{38}Cl$. A-35512 factor C has an approximate molecular weight of 1862.

A-35512 factor C dihydrochloride is a white amorphous compound. A-35512 factor C dihydrochloride has the following approximate elemental composition:

Carbon, 51.76%; hydrogen, 5.07%;
nitrogen, 5.61%; oxygen, 30.29%;
chlorine, 4.88%.

The empirical formula for A-35512 factor C dihydrochloride is in the range of $C_{83-85}H_{97-101}N_8O_{37-38}Cl_3$.

The elemental composition is in particular agreement with a possible empirical formula of $C_{84}H_{97}N_8O_{38}Cl \cdot 2HCl$ (Calcd.: C, 52.2%; H, 5.1%; N, 5.8%; O, 31.5%; Cl, 5.4%).

The infrared absorption spectrum of A-35512 factor C dihydrochloride in KBr pellet is shown in Figure 4 of the accompanying drawings. The most significant absorption maxima occur at the following frequencies (cm^{-1}): 3370 (strong), 3280 (shoulder), 3040 (shoulder), 2980 (shoulder), 2920 (weak), 1740 (weak), 1658 (strong), 1620 (weak), 1589 (medium), 1503 (strong), 1460 (weak), 1428 (medium), 1385 (weak), 1330 (weak), 1295 (medium), 1210 (strong), 1162 (medium), 1120 (weak), 1060 (strong), and 1005 (medium).

The ultraviolet absorption spectra of A-35512 factor C dihydrochloride show, in acidic and neutral methanol, an absorption maximum at 282 nm (ϵ 14,600) and, in basic methanol, an absorption maximum at 292 nm (ϵ 16,400), calculated using a molecular weight of 2000.

A ^{13}C nuclear magnetic resonance spectrum of A-35512 factor C dihydrochloride in D_2O has the following characteristics:

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No.	PPM	Height
1	172.9	2.5
2	172.2	2.0
3	171.5	2.2
4	171.0	3.9
5	169.6	2.0
6	158.6	1.8
7	157.8	3.0
8	156.5	2.1
9	156.1	2.8
10	155.6	4.2
11	154.6	3.9
12	151.1	1.5
13	143.3	1.4
14	136.0	3.2
15	135.4	2.7
16	133.2	3.7
17	128.7	2.3
18	126.5	3.1
19	124.6	2.1
20	123.9	2.7
21	121.5	2.2
22	120.1	1.2
23	118.0	1.7
24	116.5	1.2
25	107.8	2.7
26	104.5	1.9
27	101.7	1.9
28	94.5	1.0
29	75.9	3.0
30	74.3	2.0
31	73.4	2.3
32	72.1	3.5
33	70.9	4.3
34	68.7	2.9
35	67.4	71.7*
36	64.3	1.3
37	62.2	1.6
38	56.1	1.4
39	55.2	3.5
40	54.2	2.1
41	24.3	1.9
42	17.9	2.2
43	17.1	2.0
44	16.2	2.0

* dioxane standard

A-35512 factor C dihydrochloride has the following specific rotations:

$$[\alpha]_D^{25} - 161^\circ \text{ (c 1.05, H}_2\text{O)}$$

$$[\alpha]_{365}^{25} - 614^\circ \text{ (c 1.05, H}_2\text{O)}.$$

Electrometric titration of A-35512 factor C dihydrochloride in 66 vol. percent aqueous dimethylformamide indicates the presence of three titratable groups with pK_a values of approximately 7.30, 8.92, and 10.99, and the possible presence of two or more groups with pK_a values greater than 11.5.

The molecular weight of A-35512 factor C dihydrochloride, as determined by titration, is about 1982.

Amino acid analysis of acid-hydrolyzed A-35512 factor C dihydrochloride indicates that it contains at least five amino-acid residues, one of which appears to be glycine. The four remaining amino-acid residues have not yet been identified.

A-35512 factor C dihydrochloride is soluble in water, dimethyl sulfoxide, and aqueous dimethylformamide, is partially soluble in methanol and ethanol, and is insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile, and dioxane.

A-35512 factor C dihydrochloride is stable in aqueous solutions having a pH of from 3 to 10, for up to 146 hours.

A-35512 factor E is a white, amorphous, basic compound. A-35512 factor E has the following approximate elemental composition:

Carbon, 54.84%; hydrogen, 4.73%;
nitrogen, 5.26%; oxygen, 32.67%;
chlorine, 1.72%.

A-35512 factor E hydrochloride is a white amorphous compound. A-35512 factor E hydrochloride has the following approximate elemental composition:

Carbon, 52.67%; hydrogen, 4.59%;
nitrogen, 5.55%; oxygen, 33.51%;
chlorine, 3.62%.

The infrared absorption spectrum of A-35512 factor E hydrochloride in KBr pellet is shown in Figure 5 of the accompanying drawings. The most significant absorption maxima occur at the following frequencies (cm^{-1}): 3360 (strong), 3220 (shoulder), 2900 (weak), 1725 (weak), 1650 (strong), 1580 (medium), 1498 (strong), 1450 (weak), 1419 (weak), 1295 (medium), 1205 (medium), 1172 (medium), 1110 (weak), 1060 (strong), and 1000 (weak).

The ultraviolet absorption spectra of A-35512 factor E hydrochloride exhibit the following absorption maxima: in neutral methanol, 270 nm (sh) and 359 nm (ϵ 16,216); in acidic methanol, 286 nm (ϵ 18,018) and 310 nm (sh); in basic methanol, 270 nm (sh), 300 nm (ϵ 16,216), and 354 nm (ϵ 17,568), calculated using a molecular weight of 2000.

A-35512 factor E hydrochloride has the following specific rotation:

$[\alpha]_D^{25} -108.3^\circ$ (c 1, H_2O).

Electrometric titration of A-35512 factor E hydrochloride in 66 vol. % aqueous dimethylformamide indicates the presence of three titratable groups with pK_a values of approximately 6.30, 9.09, and 11.62, and the possible presence of one or two groups with pK_a values of 12.5 or greater (initial pH 5.45).

The molecular weight of A-35512 factor E hydrochloride, as determined by titration, is approximately 2018.

Amino acid analysis of acid-hydrolyzed A-35512 factor E hydrochloride indicates that A-35512 factor E contains six as-yet-unidentified amino acid residues.

A-35512 factor E hydrochloride is soluble in water, is partially soluble in methanol and ethanol, but is insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane.

A-35512 factor H is a white, amorphous, basic compound. A-35512 factor H has the following approximate elemental composition:

Carbon, 53.76%; hydrogen, 5.32%;
nitrogen, 5.53%; oxygen, 33.48%;
chlorine, 1.59%.

A-35512 factor H has an empirical formula in the range of $\text{C}_{85-87}\text{H}_{103-107}\text{N}_8\text{O}_{38-40}\text{Cl}$, a preferred tentative empirical formula of $\text{C}_{88}\text{H}_{104}\text{N}_8\text{O}_{39}\text{Cl}$, and an approximate molecular weight of 1908.

A-35512 factor H hydrochloride is a white, amorphous, hygroscopic compound. A-35512 factor H hydrochloride has the following average elemental composition:

Carbon, 53.10%; hydrogen, 5.37%;
nitrogen, 5.35%; oxygen, 30.12%;
chlorine, 3.78%.

The infrared absorption spectrum of A-35512 factor H hydrochloride in KBr pellet is shown in Figure 3 of the accompanying drawings. The most significant absorption maxima occur at the following frequencies (cm^{-1}): 3410 (strong), 3240 (shoulder), 2940 (weak), 1670 (strong), 1630 (shoulder), 1605 (strong), 1520 (strong), 1470 (weak), 1442 (weak), 1400 (weak), 1345 (shoulder), 1310 (medium), 1225 (medium), 1180 (weak), 1135 (weak), 1080 (strong), and 1020 (shoulder).

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The ultraviolet absorption spectra of A-35512 factor H hydrochloride show, in acidic and neutral methanol, an absorption maximum at 282 nm (ϵ 12,500) and, in basic methanol, an absorption maximum at 292 nm (ϵ 14,000), calculated using a molecular weight of 2000. The uv spectra of A-35512 factor H hydrochloride also show end absorption at 225 nm.

A ^{13}C nuclear magnetic resonance spectrum of A-35512 factor H hydrochloride in D_2O has the following characteristics:

No.	PPM	Height
2	177.2	2.7
3	171.6	5.2
4	170.9	5.8
5	169.6	4.7
6	158.9	3.1
7	157.6	4.3
8	156.6	3.8
9	155.6	4.1
10	155.4	3.8
11	154.3	2.4
12	151.3	1.6
13	137.7	2.0
14	136.7	2.2
15	136.0	4.0
16	135.3	1.9
17	133.5	5.0
18	129.4	3.7
19	127.3	1.3
20	126.1	3.2
21	124.2	6.9
22	122.6	4.1
23	107.6	2.7
24	101.8	1.8
25	76.2	2.8
26	73.5	4.4
27	72.3	7.4
28	71.0	12.2
29	69.7	4.6
30	67.4	73.1*
31	61.6	3.5
32	56.8	1.8
33	55.4	2.8
34	55.0	1.5
35	24.5	2.6
36	17.9	4.6
37	17.2	2.6
38	16.3	3.6

* dioxane standard

A-35512 factor H hydrochloride has the following specific rotation:

$$[\alpha]_D^{25} -123.5^\circ (c\ 1, \text{H}_2\text{O}).$$

Electrometric titration of A-35512 factor H hydrochloride in 66 vol. percent aqueous dimethylformamide indicates the presence of five titratable groups with pK_a values of approximately 5.0, 7.46, 9.80, 11.43 and 13.02 (initial pH 5.93).

The molecular weight of A-35512 factor H hydrochloride, as determined by titration, is approximately 1660.

Amino acid analysis of acid-hydrolyzed A-35512 factor H hydrochloride indicates that A-35512 factor H contains at least five amino acid residues, one of which is glycine. The four remaining amino acid residues in A-35512 factor H appear to be identical to those found in A-35512 factors A and B.

A-35512 factor H hydrochloride is soluble in water, is partially soluble in methanol and ethanol, but is insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane.

A-35512 factor H hydrochloride is stable for up to 72 hours in aqueous solutions having a pH of from 3 to 10.

A-35512 factors A, B, C, E, and H and minor factors F and G are conveniently separated by paper chromatography using a 1-butanol:pyridine:acetic acid:water (15:10:3:12) by vol. solvent system. Bioautography, using *Sarcina lutea*, is a preferred detection method and was used in this determination. The approximate R_f values of the A-35512 factors in this system are given in Table I.

TABLE I

A-35512 Factor	R_f Value
Factor A . 2HCl	0.21
Factor B . 2HCl	0.34
Factor C . 2HCl	0.46
Factor E . HCl	0.64
Factor F	0.81
Factor G	0.93
Factor H . HCl	0.15

In various systems A-35512 factor H has a chromatographic profile very similar to that of antibiotic AM374 (U.S. Patent 3,700,768). Antibiotic A-35512 factor H can be distinguished from antibiotic AM374 in at least two paper-chromatographic systems. The approximate R_f values of A-35512 factor H . 2HCl and AM374 in these two systems using *Sarcina lutea* as the detection organism are given in Table II.

TABLE II

Solvent System	R_f Value	
	A35512H	AM374
CH ₃ OH:0.1N HCl (3:1) by vol.	0.47	0.58
1-propanol:NH ₄ OH:H ₂ O (6:3:1) by vol.	0.11	0.20

Several of the A-35512 factors may be separated by high performance liquid chromatography (HPLC), using polyamide (ZIPAX, (Registered Trade Mark) Dupont) as the stationary phase and a 1.33 molar aqueous monobasic potassium phosphate solution as the mobile phase, and detecting by uv (250 nm). In Table III are given the retention times for the A-35512 factors in a representative separation by HPLC, using the following conditions:

Column Size: 1/8"x6'
 Packing: polyamide (ZIPAX, DuPont)
 Solvent: KH₂PO₄(327 g)/H₂O (1800 ml)
 Flow Rate: 0.5 ml/min
 Chart Speed: 2"/hr
 Pressure: 1500 P.S.I.

TABLE III

A-35512 Factor	Retention Time (minutes)
A	9.375
B	13.125
C	26.25
E	5.625
F	7.5
G	7.5
H	7.5

A-35512 factor B aglycone hydrochloride is a white amorphous compound having the following approximate elemental composition:

Carbon, 54.29%; hydrogen, 4.34%;
 nitrogen, 7.40%; chlorine, 5.02%;
 oxygen, 28.95% (by difference).

The infrared absorption spectrum of A-35512 factor B aglycone hydrochloride in Nujol mull is shown in Figure 6 of the accompanying drawings. The most

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significant absorption peaks occur at the following frequencies (cm^{-1}): 3440 (shoulder), 3340 (shoulder), 3215 (strong), 2950 (shoulder), 2910 (strong), 2840 (strong), 2640 (shoulder), 1735 (weak), 1655 (strong), 1590 (medium), 1500 (strong), 1460 (strong), 1378 (medium), 1365 (shoulder), 1298 (medium), 1215 (medium), 1155 (medium), 1120 (shoulder), 1105 (weak), 1060 (weak), 1040 (weak), 1008 (medium), 925 (weak), 875 (weak), 765 (shoulder), and 718 (weak).

Electrometric titration of A-35512 factor B aglycone hydrochloride in 66 vol. percent aqueous dimethylformamide indicates the presence of three titratable groups with pK_a values of approximately 7.5, 9.25, and 11.0 and the possible presence of two additional groups with pK_a values greater than 11.0.

A-35512 factor B aglycone hydrochloride has a molecular weight of about 1282, as determined by titration.

A-35512 factor B aglycone hydrochloride has the following specific rotations:

$$[\alpha]_D^{25} - 178^\circ \text{ (c 5, CH}_3\text{OH)}$$

$$[\alpha]_{365}^{25} - 716.8^\circ \text{ (c 5, CH}_3\text{OH)}$$

The ultraviolet absorption spectra of A-35512 factor B aglycone hydrochloride show, in acidic and neutral methanol, an absorption maximum at 282 nm ($E_{1\%}^{1\text{cm}}$ 102.62) and, in basic methanol, an absorption maximum at 302 nm ($E_{1\%}^{1\text{cm}}$ 182.09).

A ^{13}C nuclear magnetic resonance spectrum of A-35512 factor B aglycone in DMSO-d_6 at 90°C . has the following characteristics:

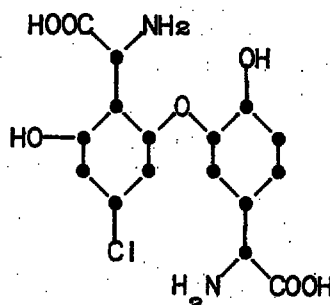
No.	PPM	Height
1	187.8	37.2
2	172.0	40.9
3	170.7	45.2
4	170.1	46.9
5	169.6	47.7
6	168.4	57.6
7	166.7	52.5
8	157.4	49.9
9	156.6	45.5
10	155.7	55.8
11	155.6	71.5
12	155.4	56.7
13	154.5	50.5
14	149.3	43.0
15	138.8	38.8
16	136.9	54.3
17	136.3	40.2
18	135.2	31.7
19	134.7	28.4
20	133.8	40.9
21	128.2	102.9
22	126.2	77.2
23	123.0	57.5
24	121.3	38.1
25	117.7	44.4
26	117.0	31.5
27	108.6	31.0
28	106.7	47.9
29	105.7	81.2
30	103.2	26.5
31	93.6	33.4
32	74.9	39.8
33	71.8	33.9
34	68.9	40.2
35	63.2	43.1
36	60.4	33.6
37	57.1	57.8
38	55.2	33.1
39	53.2	30.1
40	51.8	36.0

No.	PPM	Height
41	40.7	43.4*
42	39.9	60.9*
43	39.1	43.9*
44	23.8	55.7
45	17.1	47.8
46	0.0	43.7

* DMSO-d₆

The ¹³C nmr spectrum indicates that A-35512 factor B aglycone continues to retain the 3 - amino - 2,3,6 - trideoxy - 3 - C - methyl - L - xylo - hexopyranose moiety (one of the sugars present in A-35512 factor B).

Amino-acid analysis of further-acid-hydrolyzed A-35512 factor B aglycone hydrochloride indicates that A-35512 factor B aglycone contains glycine and at least three complex amino acid residues. The structure of one of these amino acid residues appears to be:



A-35512 factor B aglycone hydrochloride has at least one hydroxyl group capable of esterification.

A-35512 factor B aglycone hydrochloride is soluble in water and methanol, but is insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane.

A-35512 factor B aglycone hydrochloride has an approximate R_f value of 0.80 on cellulose thin-layer chromatography (aluminum support), using a 1-butanol:pyridine:acetic acid:water (15:10:3:12) by vol. solvent system. Bioautography, using *Sarcina lutea*, is a preferred detection method, and was used in this determination.

A-35512 factor B aglycone hydrochloride has an approximate R_f value of 0.26 on silica gel thin-layer chromatography using a methanol:chloroform:conc. NH₄OH (3:2:1) by vol. solvent system.

A-35512 factor B aglycone (free base) is a white amorphous compound having the following approximate elemental composition:

Carbon, 52.65%; hydrogen, 4.57%;
nitrogen, 6.91%; chlorine, 2.94%;
oxygen, 27.04%; ash, 4.70%.

The infrared absorption spectrum of A-35512 factor B aglycone (free base) in KBr pellet exhibits significant absorption peaks at the following frequencies (cm⁻¹): 3360 (strong), 3260 (shoulder), 2940 (shoulder), 1735 (shoulder), 1660 (strong), 1598 (medium), 1510 (strong), 1440 (medium), 1295 (weak), 1215 (medium), 1165 (medium), 1122 (weak), 1070 (weak), 1018 (strong), 940 (weak), and 920 (weak).

Electrometric titration of A-35512 factor B aglycone (free base) in 66 vol. % aqueous dimethylformamide indicates the presence of five titratable groups with pK_a values of approximately 6.2, 8.2, 10.1, 11.4, and 12.4 and the possible presence of one or two additional groups with pK_a values greater than 12.5.

A-35512 factor B aglycone (free base) has the following specific rotation:

[α]_D²⁵ -64.5° (c 3, DMSO).

The ultraviolet absorption spectra of A-35512 factor B aglycone (free base) show, in neutral and acidic methanol, an absorption maximum at 282 nm (E_{1%^{1cm}} 43.65) and, in basic methanol, an absorption maximum at 301 nm (E_{1%^{1cm}} 67.46).

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A-35512 factor B aglycone (free base) has the same approximate R_f values earlier-described for A-35512 factor B aglycone hydrochloride.

In addition to the free base and hydrochloride forms of the individual A-35512 factors and A-35512 factor B aglycone other pharmaceutically-acceptable acid-addition salts of A-35512 factors A, B, C, E, H and factor B aglycone are also part of this invention. "Pharmaceutically-acceptable" salts are salts in which the toxicity of the compound as a whole toward warm-blooded animals is not increased relative to the non-salt form. Representative and suitable salts of A-35512 factors A, B, C, E, and H and A-35512 factor B aglycone include those salts formed by standard reaction with both organic and inorganic acids for example, sulfuric, phosphoric, acetic, succinic, citric, lactic, maleic, fumaric, palmitic, cholic, pantoic, mucic, D-glutamic, d-camphoric, glutaric, glycolic, phthalic, tartaric, lauric, stearic, salicylic, methanesulfonic, benzenesulfonic, sorbic, picric, benzoic and cinnamic acids.

The novel antibiotics of this invention are produced by culturing an A-35512-producing strain of *Streptomyces candidus* NRRL 8156 under submerged aerobic conditions in a suitable culture medium until substantial antibiotic activity is produced. The antibiotics are recovered by employing various isolation and purification procedures used in the fermentation art.

The new organism useful for the preparation of A-35512 antibiotics was isolated from a soil sample collected on Eniwetok Atoll. This organism is classified as a novel strain of *Streptomyces candidus* (Krassilnikov) Waksman, as described by E. B. Shirling and D. Gottlieb in "Cooperative Description of Type Cultures of *Streptomyces*. II. Species Descriptions from Second Study," *Intern. J. Systematic Bacteriol.* 18(4):279-392 (1968); and S. A. Waksman, "The Actinomycetes. Vol. 2. Classification, Identification, and Descriptions of Genera and Species," Williams and Wilkins Co., Baltimore, 1961.

This classification is based on methods recommended for the International *Streptomyces* Project [E. B. Shirling and D. Gottlieb, "Methods for Characterization of *Streptomyces* Species," *Intern. Bull. Systematic Bacteriol.* 16:313-340 (1966)] along with certain supplementary tests. Color names were assigned according to the ISCC-NBS method (K. L. Kelly and D. B. Judd, "The ISCC-NBS Method of Determining Colors and a Dictionary of Color Names," U.S. Department of Commerce Cir. 553, Washington, D.C., 1955). Figures in parentheses refer to the Tresner and Backus color series [H. D. Tresner and S. J. Backus, "System of Color Wheels for Streptomycete Taxonomy," *Appl. Microbiol.* 11:335-338 (1963)] and color tab designations are underlined. The Maerz and Paul color blocks (A. Maerz and M. R. Paul, "Dictionary of Color," McGraw-Hill, New York, N.Y., 1950) are enclosed in brackets. Cultures were grown at 30°C. for 14 days unless otherwise noted.

Morphology

Long, wavy sporophores are produced. Spores, which occur in chains of 10-50, are cylindrical and measure from 0.7 to 1.05 μ x 1.4 to 3.5 μ . Sclerotia-like structures are produced on several media. Broom-shaped or fascicled hyphae were also observed occasionally. The spore surface, as observed by electron microscopy, is smooth.

Cultural Characteristics on Various Media

Medium	Characteristics	
ISP No. 2 (yeast extract-malt extract agar)	Growth-abundant; reverse grayish yellow [11J5]; abundant aerial mycelium and sporulation, white (w) a; no soluble pigment.	50
ISP No. 3 (oatmeal agar)	Growth-moderate; reverse pale yellow green [10B1]; fair aerial mycelium and sporulation, white (w) 13ba; no soluble pigment; sclerotia-like bodies observed.	55
ISP No. 4 (inorganic salts-starch agar)	Growth-good; reverse amber white [10C1]; good aerial mycelium and sporulation, yellowish gray (GY) 2dc; slight brown soluble pigment; sclerotia-like bodies observed.	60

Cultural Characteristics on Various Media

	Medium	Characteristics	
5	ISP No. 5 (glycerol asparagine agar)	Growth-good; reverse pale yellow green [10B2]; good aerial mycelium and sporulation, white (w) <i>a</i> ; no soluble pigment; mycelium appears to be fascicled.	5
10	Emerson's agar	Growth-good; reverse light olive [14L6]; no aerial mycelium or sporulation; slight brown soluble pigment.	10
15	Bennet's modified agar	Growth-abundant; reverse moderate yellow [11J6]; abundant aerial mycelium and sporulation, white (w) <i>a</i> ; no soluble pigment.	15
20	Czapek's-solution agar	Growth-good; reverse pale yellow green [10B1]; good aerial mycelium and sporulation, white (w) <i>b</i> ; no soluble pigment; mycelium appears fascicled.	20
25	Tomato-paste oatmeal agar	Growth-abundant; reverse grayish yellow [11I5]; abundant aerial mycelium and sporulation, white (w) <i>a</i> ; mycelium "rolls up" away from agar surface; no soluble pigment.	25
30	Nutrient agar	Growth-fair to good; reverse pale yellow green [18D2]; good aerial mycelium and sporulation, white (w) <i>a</i> ; slight brown soluble pigment.	30
35	Glucose asparagine agar	Growth-good; reverse pale yellow green [17E1]; good aerial mycelium and sporulation, white (w) <i>13ba</i> ; no soluble pigment.	35
40	Tryptone yeast-extract agar	Growth-fair; reverse white [10A1]; fair aerial mycelium and sporulation, white (w) <i>b</i> ; no soluble pigment.	40
45	Tyrosine agar	Growth-good, reverse white [10B2]; good aerial mycelium and sporulation, white (w) <i>a</i> ; brown soluble pigments; sclerotia-like bodies observed.	45
50	Glycerol glycine agar	Growth-good; reverse white [10B2]; good aerial mycelium and sporulation, white (w) <i>b</i> ; slight brown soluble pigment.	50
55	Calcium malate agar	Growth-good; reverse white [10B1]; good aerial mycelium and sporulation, white (w) <i>a</i> ; no soluble pigment; medium cleared around area of inoculum.	55

The organism was studied for selected physiological properties in accordance with standard procedures. The properties observed and characteristics found were as follows:

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	Property Observed	Characteristics	
	Action on milk	Coagulation with some clearing at 14 days.	
5	Nitrate reduction	Positive	5
	Melanin-pigment production on:		
	Peptone iron agar slants	Negative	
	Tyrosine agar slants	Weak Positive (pigment after 7 days)	
10	Tryptone yeast extract broth	Negative	10
	Gelatin liquefaction	Complete at 14 days	
	Temperature requirements:	26—30°C.-good growth and sporulation	
15	(ISP medium No. 2; yeast-extract malt-extract slants)	37°C.-sparse growth; no aerial mycelium or spores	15
		40°C.-slight vegetative growth only	
		45°C.-no growth	

20	The results of carbon utilization tests carried out with organism NRRL 8156 are set forth below. The symbols used to indicate growth response are:	20
	+ = good growth, positive utilization	
	(+) = poor to fair growth	
	(-) = faint growth, probably no utilization	
25	- = no growth, no utilization	25

	Carbon Source		
	none (negative control)	(-)	
	D-glucose (positive control)	+	
30	L-arabinose	(-)	30
	sucrose	(-)	
	D-inositol	+	
	D-mannitol	+	
	D-fructose	+	
35	rhamnose	(-)	35
	raffinose	(-)	
	D-xylose	(+)	

40	Certain characteristics of the A-35512-producing <i>S. candidus</i> NRRL 8156 strain differ from the characteristics of the organism described by Shirling and Gottlieb [Elwood B. Shirling and David Gottlieb, "Cooperative Description of Type Cultures of <i>Streptomyces</i> III. Additional Species Descriptions from First and Second Studies," <i>Intern. J. Systematic Bacteriol.</i> 18(2), 69—189 (1968)] and by Waksman [S. A. Waksman, "The Actinomycetes. Classification, Identification and Descriptions of Genera and Species," Vol. 2, The Williams and Wilkins Co., Baltimore, Md., 1961]. These differences are summarized in Table IV:	40
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45	TABLE IV	45
	NRRL 8156	Published Description
50	Carbon Utilization	
	L-arabinose	+
	rhamnose	+
	D-inositol	-
	Gelatin Liquefaction	Complete at 14 days
55	Action on Milk	No coagulation; good peptonization
		Coagulation; some clearing at 14 days

The *Streptomyces candidus* culture useful for the production of A-35512 antibiotics has been deposited and made a part of the stock culture collection of the Northern Regional Research Center, U.S. Department of Agriculture, Agricultural Research Service, Peoria, Illinois, 61604, from which it is available to the public under the number NRRL 8156.

As in the case with other organisms, the characteristics of the A-35512-producing culture, *Streptomyces candidus* NRRL 8156, are subject to variation. For example, artificial variants and mutants of the NRRL 8156 strain may be obtained by treatment with various known mutagens such as ultraviolet rays, X-rays, high-frequency waves, radioactive rays and chemicals. All natural and artificial variants and mutants which belong to *Streptomyces candidus* and produce the A-35512 antibiotics may be used in this invention.

The culture medium employed to grow *Streptomyces candidus* NRRL 8156 can be any one of a number of media. For economy in production, optimal yield, and ease of product isolation, however, certain culture media are preferred. Thus, for example, a preferred carbohydrate source in large-scale fermentation is sucrose, although glucose, tapioca dextrin, fructose, mannitol, maltose and lactose may also be employed. A preferred nitrogen source is soluble meat peptone, although soybean flour, pork-blood meal, amino acids such as glutamic acid are also useful. Among the nutrient inorganic salts which can be incorporated in the culture media are the customary soluble salts capable of yielding zinc, sodium, magnesium, calcium, ammonium, chloride, carbonate, sulfate and nitrate ions.

Essential trace elements necessary for the growth and development of the organism should also be included in the culture medium. Such trace elements commonly occur as impurities in other constituents of the medium in amounts sufficient to meet the growth requirements of the organism.

It may be necessary to add small amounts (i.e. 0.2 ml/l.) of an antifoam agent such as polypropylene glycol to large-scale fermentation media if forming becomes a problem.

For production of substantial quantities of the A-35512 antibiotics, submerged aerobic fermentation in tanks is preferred. Small quantities of the A-35512 antibiotics may be obtained by shake-flask culture. Because of the time lag in antibiotic production commonly associated with inoculation of large tanks with the spore form of the organism, it is preferable to use a vegetative inoculum. The vegetative inoculum is prepared by inoculating a small volume of culture medium with the spore form or mycelial fragments of the organism to obtain a fresh, actively growing culture of the organism. The vegetative inoculum is then transferred to a larger tank.

The A-35512-producing organism is preferably grown at temperatures from 20° to 40°C; most preferably A-35512 production appears to occur at temperatures of 30°—34°C.

As is customary in aerobic submerged culture processes, sterile air is blown through the culture medium. For efficient growth of the organism the volume of air employed in tank production is preferably above 0.1 volume of air per volume of culture medium per minute (V/V/M). For efficient production of the A-35512 antibiotics the volume of air employed in tank production is preferably about 0.25 V/V/M.

Production of the A-35512 antibiotics can be followed during fermentation by testing samples of the broth or of extracts of the mycelial solids for antibiotic activity against organisms known to be sensitive to the antibiotics. One assay organism useful in testing these antibiotics is *Bacillus subtilis* ATCC 6633. The bioassay is preferably performed by paper-disc assay on agar plates containing a nutritionally-limited medium.

Following their production under submerged aerobic fermentation conditions, the A-35512 antibiotics can be recovered from the fermentation medium by methods employed in the fermentation art. The antibiotic activity produced during fermentation of an A-35512-producing organism generally occurs in the filtered broth. Maximum recovery of the A-35512 antibiotics is accomplished, therefore, by an initial filtration to remove the mycelial mass. The filtered broth can be purified to give the A-35512 mixture by a variety of techniques. A preferred technique involves adsorption of the filtered broth on a polyamide column and elution of the column with water and aqueous alcohol mixtures. The eluted fractions which exhibit antibiotic activity can be combined to give the A-35512 mixture. Alternatively, using the technique, the eluted fractions can be combined on the

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basis of thin-layer-chromatographic behavior to give purified A-35512 factor B and enriched mixtures of the other A-35512 factors.

Further purification of the individual A-35512 factors includes additional adsorption and extraction procedures. Adsorptive materials such as alumina, silica gel, ion-exchange resins can be advantageously used.

The A-35512 factors occur in the fermentation broth as hydrochlorides. The preferred polyamide separation procedure provides A-35512 factor B and the remaining portion of the A-35512 mixture as hydrochlorides. Each individual factor may be converted to its free base (ionic chlorine free) form by accepted procedures, for example, chromatography over a weakly-basic ion-exchange resin.

Alternatively, the culture solids, including medium constituents and mycelium can be used without extraction or separation, but preferably after removal of water, as a source of the A-35512 antibiotics. For example, after production of A-35512 antibiotic activity, the culture medium can be dried by lyophilization and mixed directly into feed premix.

A-35512 factor B aglycone may be prepared by acid hydrolysis of A-35512 factor B. A-35512 factor B is most readily available in its dihydrochloride form. A-35512 factor B dihydrochloride is, therefore, a preferred starting material for the preparation of A-35512 factor B aglycone. A-35512 factor B or other A-35512 factor B acid addition salts may also be used. The acid hydrolysis is carried out according to standard procedures. Although any one of a number of acids may be used, hydrochloric acid is a preferred acid for the preparation of A-35512 factor B aglycone. When hydrochloric acid is used, the A-35512 factor B aglycone will be recovered as a hydrochloride salt. The hydrolysis is preferably carried out in water under reflux for a period of from one to two hours. Longer reaction times result in degradation of the aglycone to give less active, and, later, inactive products. Optimum reaction times for specific reaction conditions can be determined by checking reaction aliquots for bioactivity.

The A-35512 antibiotic mixture, A-35512 antibiotic factors A, B, C, E and H and A-35512 factor B aglycone inhibit the growth of certain pathogenic organisms, particularly gram-positive bacteria. The minimal inhibitory concentrations (MIC's) at which the A-35512 factors A, B, C and H inhibit selected bacteria, as determined by standard agar-dilution tests, are summarized in Table V.

TABLE V

Test Organism	MIC (mcg/ml)			
	A-35512 factor A . 2HCl	A-35512 factor B . 2HCl	A-35512 factor C . 2HCl	A-35512 factor H . HCl
<i>Staphylococcus aureus</i> 3055	6.25	3.12	6.25	25.0
<i>Staphylococcus aureus</i> 3074	25.0	6.25	6.25	25.0
<i>Streptococcus faecalis</i>	6.25	3.12	3.12	12.5

[The *Staphylococcus aureus* numbers are laboratory-assigned reference numbers]

In one important aspect, the A-35512 antibiotics inhibit the growth of organisms which are resistant to other antibiotics. In Table VI is summarized the standard-disc-plate-assay activity of A-35512 factors A, B, C, E and H (30 mcg/disc) against representative organisms. Activity is measured as the diameter (in mm) of the observed zones of inhibition.

TABLE VI

	Test Organism	Zone Diameters (mm)					
		A-35512 factor A . 2HCl	A-35512 factor B . 2HCl	A-35512 factor C . 2HCl	A-35512 factor E . HCl	A-35512 factor H . HCl	
5	<i>Staphylococcus aureus</i> 3055 (penicillin G susceptible)	13.4	18.4	0	14.4	11.9	5
	<i>Staphylococcus aureus</i> 3074 (penicillin G resistant)	13.2	16.8	0	14.5	11.6	
10	<i>Staphylococcus aureus</i> 3130 (methicillin resistant)	14.4	18.4	0	15.4	12.6	10
	<i>Streptococcus pyogenes</i> (Group A)	17.0	19.6	10.5	17.0	16.0	
15	<i>Streptococcus sp.</i> 9960 (Group D)	15.5	19.4	0	16.4	13.8	15
	<i>Diplococcus pneumoniae</i> Park I	—	—	11.0	20.0	—	

[The *Staphylococcus aureus* and *Streptococcus sp.* numbers are laboratory assigned reference numbers]

20 Table VII gives additional agar-dilution test results for A-35512 factor B dihydrochloride, giving the minimal inhibitory concentrations (MIC) against several Group A Streptococci and *Diplococcus pneumoniae*. 20

TABLE VII

		MIC (mcg/ml)	
	Test Organism	A-35512 factor B	
25	<i>Streptococcus pyogenes</i> C203	1	25
	<i>Streptococcus pyogenes</i> 10389	1	
	<i>Streptococcus pyogenes</i> 12344	8	
30	<i>Streptococcus pyogenes</i> 12961	1	30
	<i>Streptococcus pyogenes</i> 663—72	0.5	
	<i>Streptococcus pyogenes</i> 664—72	0.5	
	<i>Streptococcus pyogenes</i> 665—72	1	
	<i>Streptococcus pyogenes</i> 13234	0.5	
35	<i>Streptococcus pyogenes</i> 19035	0.5	35
	<i>Streptococcus pyogenes</i> M6517	0.5	
	<i>Streptococcus pyogenes</i> D27781	1	
	<i>Diplococcus pneumoniae</i> Park I	2	
	<i>Diplococcus pneumoniae</i> Type 14	2	

40 [The organisms numbers are laboratory assigned reference numbers] 40

Table VIII gives additional agar-dilution test results for A-35512 factor B dihydrochloride against several Group D Streptococci.

TABLE VIII

45		MIC (mcg/ml)	45
	Test Organism	A-35512	
		factor B	
	<i>Streptococcus sp.</i> D282	2	
	<i>Streptococcus sp.</i> 9901	2	
50	<i>Streptococcus sp.</i> 9913	4	50
	<i>Streptococcus sp.</i> 9933	4	
	<i>Streptococcus sp.</i> 9960	4	
	<i>Streptococcus sp.</i> 12253F	4	
	<i>Streptococcus sp.</i> Shrigley	4	
	<i>Streptococcus sp.</i> Mitis	4	
55	<i>Streptococcus sp.</i> 238	2	55
	<i>Streptococcus sp.</i> SS992	4	

[The organism numbers are laboratory assigned reference numbers]

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Table IX provides additional agar-dilution test results for A-35512 factor B dihydrochloride against other gram-positive organisms.

TABLE IX

MIC (mcg/ml)
A-35512
factor B

Test Organism	
<i>Staphylococcus aureus</i> 3123*	4
<i>Staphylococcus aureus</i> H43**	4
<i>Staphylococcus aureus</i> 3124**	4
<i>Staphylococcus aureus</i> 3126**	2
<i>Staphylococcus aureus</i> 3127**	4
<i>Staphylococcus aureus</i> H57**	2
<i>Staphylococcus aureus</i> 3074**	4
<i>Staphylococcus aureus</i> 3130***	4
<i>Staphylococcus aureus</i> 3131****	4
<i>Staphylococcus aureus</i> 3133***	2
<i>Staphylococcus aureus</i> 3136***	2
<i>Staphylococcus aureus</i> 3125***	4
<i>Staphylococcus epidermis</i> 3064**	2
<i>Staphylococcus epidermis</i> 3078*	2
<i>Bacillus subtilis</i> ATCC 6633	2
<i>Sarcina lutea</i> PCI-1001-FDa	1

* penicillin-G susceptible

** penicillin-G resistant

*** penicillin-G resistant; methicillin resistant

**** penicillin-G resistant; methicillin resistant; clindamycin resistant

[The organism numbers are laboratory assigned reference numbers]

The A-35512 antibiotics also inhibit the growth of certain anaerobic bacteria. Table X summarizes the activity of A-35512 factor B dihydrochloride against various anaerobic bacteria, using the standard agar-dilution test.

TABLE X

MIC (mcg/ml)
A-35512
factor B

Test Organism	
<i>Actinomyces israelii</i>	0.5
<i>Clostridium perfringens</i>	2.0
<i>Clostridium septicum</i>	4.0
<i>Eubacterium aerofaciens</i>	2.0
<i>Peptococcus asaccharolyticus</i>	1.0
<i>Peptococcus prevoti</i>	2.0
<i>Peptostreptococcus anaerobius</i>	1.0
<i>Peptostreptococcus intermedius</i>	4.0
<i>Propionibacterium acnes</i>	0.5
<i>Bacteroides fragilis</i> ssp. <i>fragilis</i> 111	64.0
<i>Fusobacterium necrophorum</i>	8.0

Antibiotic A-35512 factor B dihydrochloride has shown *in vivo* antimicrobial activity against experimental bacterial infections. When two subcutaneous doses of A-35512 factor B were administered to mice in illustrative infections, the activity observed was measured as an ED₅₀ value [effective dose in mg/kg to protect 50 percent of the test animals; see Warren Wick, *et al.*, *J. Bacteriol.* 81, 233-235 (1961)]. The ED₅₀ values observed for A-35512 factor B dihydrochloride are given in Table XI.

TABLE XI

Test Organism	ED ₅₀	Infecting Challenge
<i>Streptococcus pyogenes</i> C203	1.75	260×LD ₅₀ (ip)
<i>Diplococcus pneumoniae</i> Park I	4.3	60.8×LB ₅₀ (ip)
<i>Staphylococcus aureus</i> 3055	6.9	206×LD ₅₀ (ip)

A-35512 factor B dihydrochloride also provided protection to guinea pigs which had been infected with the anaerobe *Clostridium chauvoei* (the disease agent of Black leg in the bovine). These tests were carried out according to the U.S.D.A. Supplemental Assay Method for Potency Testing *Clostridium chauvoei*-containing Products (SAM 200, U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Veterinary Services Laboratories, Ames, Iowa 50010, April 7, 1975, under 9 C.F.R. 113.91). The test results are given in Table XII.

TABLE XII

Treatment	Challenge Dead/Total	Percent Protected
A-35512 Factor B 10 mg/kg*	0/10	100
A-35512 Factor B 1 mg/kg*	3/10	70
Challenge Control	10/10	0

* Intramuscular administration

The activity of A-35512 factor B aglycone, as measured by convention disc-diffusion assay (6 mm pads dipped in solutions containing 1 mg of test compound per ml of solution and placed on agar plates seeded with test organism), is summarized in Table XIII.

TABLE XIII

Test Organism	Zone of Inhibition (mm) A-35512 factor B aglycone (free base)
<i>Staphylococcus aureus</i>	19
<i>Bacillus subtilis</i>	13
<i>Sarcina lutea</i>	14
<i>Bacillus subtilis</i> *	22

* With nutritionally-limited agar

Table XIV summarizes minimal inhibitory concentrations (MIC's) at which A-35512B aglycone hydrochloride is active against selected *Staphylococcus aureus* strains, as determined by standard agar-dilution tests.

TABLE XIV

Test Organism	MIC (mcg/ml)
<i>Staphylococcus aureus</i> 3055*	0.5
<i>Staphylococcus aureus</i> H290*	0.5
<i>Staphylococcus aureus</i> V92*	0.5
<i>Staphylococcus aureus</i> V104*	0.25
<i>Staphylococcus aureus</i> 3074**	0.25
<i>Staphylococcus aureus</i> H43**	0.5
<i>Staphylococcus aureus</i> V57**	0.5
<i>Staphylococcus aureus</i> H356**	1.0
<i>Staphylococcus aureus</i> 3130***	0.5
<i>Staphylococcus aureus</i> 3131****	0.5

* penicillin-G susceptible

** penicillin-G resistant

*** penicillin-G resistant; methicillin resistant

**** penicillin-G resistant; methicillin resistant; clindamycin resistant

[The organism numbers are laboratory-assigned reference numbers]

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Table XV gives additional agar-dilution test results for A-35512 factor B aglycone hydrochloride against several Group-D streptococci.

TABLE XV

	Test Organism	MIC (mcg/ml)	
5	<i>Streptococcus</i> sp. Shrigley	1.0	5
	<i>Streptococcus</i> sp. Mitis	1.0	
	<i>Streptococcus</i> sp. 12253F	1.0	
	<i>Streptococcus</i> sp. SS992	1.0	
	<i>Streptococcus</i> sp. 9933	1.0	
10	<i>Streptococcus</i> sp. 9913	1.0	10
	<i>Streptococcus</i> sp. 282	1.0	
	<i>Streptococcus</i> sp. 238	1.0	

[The organism numbers are laboratory-assigned reference numbers]

15 A-35512 factor B aglycone has shown *in vivo* antibacterial activity against experimental bacterial infections. When two subcutaneous doses of A-35512 factor B aglycone hydrochloride were administered to mice in illustrative infections, the observed activity was measured as an ED₅₀ value. The ED₅₀ values observed for A-35512 factor B aglycone hydrochloride are given in Table XVI. 15

TABLE XVI

	Test Organism	ED ₅₀	Infecting Challenge	
20	<i>Streptococcus pyogenes</i> C203	5.8	2570×LD ₅₀ (ip)	20
	<i>Streptococcus pneumoniae</i>	7.0	374×LD ₅₀ (ip)	
	<i>Staphylococcus aureus</i> 3055	1.04	370×LD ₅₀ (ip)	

[The organism numbers are laboratory-assigned reference numbers]

25 The A-35512 antibiotics are relatively non-toxic. For example, the LD₅₀ of A-35512 factor A dihydrochloride, when given subcutaneously to fasted Cox and Harlan female ICR mice, is greater than 8000 mg/kg. The LD₅₀ of A-35512 factor B dihydrochloride in mice, when given by intraperitoneal injection, is approximately 1356 mg/kg and, when given intravenously, is between 1000 and 12500 mg/kg. Both A-35512 factor C dihydrochloride and A-35512 factor E hydrochloride have an acute toxicity, when given intraperitoneally to mice, which is greater than 300 mg/kg. 25

30 Another important property of the A-35512 mixture and compounds is the ability to improve feed-utilization efficiency in animals. This ability has been demonstrated in ruminants which have a developed rumen function. 30

35 It is known that the efficiency of carbohydrate utilization in ruminants is increased by treatments which stimulate the animals' rumen flora to produce propionate compounds rather than acetate or butyrate compounds (for a more complete discussion, see Church *et al.* in "Digestive Physiology and Nutrition of Ruminants," Vol. 2, 1971, pp. 622 and 625). 35

40 The efficiency of feed use can be measured by *in vivo* tests in fistulated cattle, using the method described by Arthur P. Raun in U.S. Patent 3,794,732 (see especially Example 8). Table XVII summarizes the results of such a test with A-35512 factor B dihydrochloride wherein molar percent ruminal propionic acid concentrations were averaged over five analyses in a 21-day treatment period. 40

TABLE XVII

	Treatment	No. Animals	Average Molar Percent Propionic Acid	Molar Percent Increase over Control	
50	Control	5	19.3	—	50
	A-35512 factor B 50 g/ton	5	26.0	6.7	

55 The A-35512 mixture and compounds are typically effective in increasing propionates and, thereby, the efficiency of feed utilization when administered to ruminants orally at rates of from 0.15 to 10.0 mg/kg/day. Most beneficial results are 55

achieved at rates of from 1.0 to 2.0 mg/kg/day. A preferred method of administration of the A-35512 mixture or compound is by mixing it with the animals' feed; however, it can be administered in other ways, for example, tablets, drenches, boluses or capsules. Formulation of these various dosage forms can be accomplished by methods well known in the veterinary pharmaceutical art. Each individual dosage unit would contain a quantity of A-35512 mixture or compound directly related to the proper daily dose for the animal to be treated.

The A-35512 mixture and compounds are also useful as growth-promoting agents in animals. In tests using broiler chicks, A-35512 factor B dihydrochloride added to the feed at a rate of 20 grams per ton improved weight gains and increased feed efficiency. Table XVIII summarizes the results of such tests in a battery study using four replicates of eight birds for each of two time replicates.

TABLE XVIII

Treatment	Conc. (g/ton)	Weight Gain (g)	% Improve- ment over Control	Feed Efficiency	% Improve- ment over Control
Control	—	380	—	1.850	—
A-35512 Factor B	20	394	3.68	1.776	4.00

Table XIX summarizes the results of such tests in a floor-pen study using twelve replicates of 80 birds each.

TABLE XIX

Treatment	Conc. (g/ton)	Weight Gain (g)	% Improve- ment over Control	Feed Efficiency	% Improve- ment over Control
Control	—	1898	—	2.250	—
A-35512 Factor B	20	1956*	3.06	2.177*	2.81

* $P \leq 0.05$

The A-35512 mixture and compounds are typically effective in promoting growth in poultry when administered with the animals' feed at rates of from one to 100 grams of A-35512 mixture or compound per ton of animal feed.

In order to illustrate more fully the operation of this invention, the following examples are provided.

EXAMPLE 1

A. Shake-flask Fermentation of A-35512

A lyophilized pellet of *Streptomyces candidus* NRRL 8156 was dissolved in 1—2 ml of sterilized water. This solution was used to inoculate a Bacto yeast malt extract (ISP No. 2, Difco Laboratories, Detroit, Michigan) agar slant.

The inoculated slant was incubated at 30°C. for about seven days. The mature slant culture was covered with water (2 ml) and scraped with a sterile pipette to loosen the spores. A portion (0.1 ml) of this water suspension of spores was used to inoculate another ISP No. 2 agar slant. This inoculated slant was incubated at 30°C. for about seven days. The mature slant culture was covered with water (5 ml) and scraped with a sterile pipette to loosen the spores. A portion (2.5 ml) of the resulting suspension of spores was used to inoculate 50 ml of a vegetative medium having the following composition:

Ingredient	Amount
Trypticase Soy Broth (Baltimore Biological Laboratories, Cockeysville, Md.)	30 g
Water (deionized)	q. s. 1 liter

The inoculated vegetative medium was incubated in a 250 ml Erlenmeyer flask at 30°C. for 48 hours on a shaker rotating through an arc two inches in diameter at 250 rpm.

This incubated vegetative medium (0.5 ml) was used to inoculate 50 ml of a production medium having the following composition:

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	Ingredient	Amount (g/l.)	
	Tapioca dextrin	25.0	
	Glucose	10.0	
5	NH ₄ NO ₃	2.5	
	KCl	1.5	5
	MgSO ₄	1.1	
	FeCl ₂ · 4H ₂ O	.03	
	ZnCl ₂	.03	
	KH ₂ PO ₄	0.1	
10	L-Glutamic acid	1.0	10
	DL-citrulline	0.1	
	CaCO ₃	5.0	
	Deionized water	q.s. 1 liter	

15 The inoculated production medium was incubated in a 250 ml Erlenmeyer flask at 32°C. for 8—10 days on a shaker rotating through an arc two inches in diameter at 250 rpm. 15

B. Tank Fermentation of A-35512

20 In order to provide a larger volume of inoculum, 20 ml of incubated vegetative medium prepared as described above was used to inoculate 400 ml of a second-stage vegetative growth medium having the same composition as that of the vegetative medium. This second-stage medium was incubated in a 2-liter flask for 24 hours at 32°C. on a shaker rotating through an arc 2 inches in diameter at 250 rpm. 20

25 Incubated second-stage vegetative medium (800 ml) thus prepared was used to inoculate 100 liters of sterile production medium. The inoculated production medium was allowed to ferment in a 165-liter fermentation tank for 8—10 days at a temperature of 32°C. The fermentation medium was aerated with sterile air at the rate of 0.25 V/V/M and was stirred with conventional agitators at 200 rpm. 25

EXAMPLE 2

30 Incubated vegetative medium prepared as described in Example 1, Section A, can alternatively be stored for later use by maintaining in the vapor phase of liquid nitrogen by the following procedure: 30

In a small (13×100-mm) sterilized screw-cap tube is placed 2 ml of a suspension agent having the following composition:

	Ingredient	Amount	
35	Glycerol	20% by weight	35
	Lactose	10% by weight	
	Water (deionized)	70% by weight	

40 To this suspension agent is added 2 ml of a 48-hour incubated vegetative medium prepared as above-described. The mixed solution is frozen and maintained in the gas phase of a liquid-nitrogen tank. 40

45 Vegetative medium thus stored is thawed for use in shake or tank fermentation by placing the vial in a 43°C. water bath. A portion of the thawed solution (1 ml) in the vial is used to inoculate 50 ml of a vegetative medium having the same composition as that described in Example 1, section A. The inoculated vegetative medium is used, as described in Example 1, either for shake-flask fermentation or to provide a larger inoculum for tank fermentation. 45

EXAMPLE 3

50 The fermentation is carried out according to the method of Example 1, but using a shake-flask/tank production medium having the following composition: 50

	Ingredient	Amount (g/l.)	
	Tapioca dextrin	75.0	
	Molasses	40.0	
	Soluble meat peptone	15.0	
55	MgSO ₄ · 7H ₂ O	0.5	55
	CaCO ₃	2.0	
	Water	q.s. 1 liter	

EXAMPLE 4

Separation of A-35512 Antibiotic Mixture

Whole fermentation broth (250 gal.), obtained as described in Example 1, was filtered, using a filter aid (Hyflo Super-cel, (Registered Trade Mark), a diatomaceous earth, Johns-Manville Products Corp.) at broth pH (pH 6.8—7.2). The clear filtrate thus obtained was passed through a column containing 10 ml of polymeric adsorbent (Amberlite (Registered Trade Mark) XAD-4, Rohm and Haas Co.) per 100 ml of broth filtrate at a rate of 150 ml per minute. The fractions thus obtained were monitored for biological activity using a standard disc assay against *Sarcina lutea*. The biologically inactive effluent was discarded. The column was washed with water (1/8 of the broth volume) at a rate of 150 ml per minute. The inactive water wash was discarded.

The column was then eluted with a 50 vol. percent aqueous methanol solution (600 liters) at a rate of 200 ml per minute. The eluate, containing the A-35512 antibiotic mixture, was concentrated under vacuum to a volume of 15 liters, containing about 200 grams of A-35512 antibiotic mixture per liter.

EXAMPLE 5

Separation of Factors from A-35512 Antibiotic Mixture

The A-35512 antibiotic mixture (about 3000 grams dissolved in 15 liters of methanol), obtained as described in Example 4, was chromatographed on a polyamide column (Woelm, 100 liter). The column was eluted with deionized water at a flow rate of 80—120 ml per minute.

Fractions were monitored using cellulose thin-layer chromatography on paper chromatography, *n*-butanol:pyridine:acetic acid:water (15:10:3:12) by vol. solvent system, and *Sarcina lutea* bioautography.

The first 100 liters of eluate were discarded. The flow rate was then changed to 160—200 ml per minute, and 12 liter fractions were collected. Twenty fractions were collected in this manner.

At this point the eluting solvent was changed to a water-methanol gradient using the following procedure:

A container holding 360 liters of methanol was siphoned into a container holding 120 liters of water. In the water container the mixing solution was stirred and fed into the column. Twenty-four fractions (24 liters each) were collected at a flow rate of 200—300 ml/minute.

On the basis of bioautography results, groups of fractions were combined and evaporated to dryness under vacuum to give A-35512 factor B dihydrochloride and the following enriched mixtures of factors:

Fractions	Vol. (liters)	Factor(s)	Weight
1—10	120	A+H	192 g.
11—24	216	B	269 g.
25—31	168	B+C	590 g.
32—44	312	C, E, F, G	224 g.

EXAMPLE 6

Purification of A-35512 Factor B

Partially purified A-35512 factor B dihydrochloride (400 g.), obtained as described in Example 5, was dissolved in 1.2 liters of 50 percent aqueous methanol and chromatographed on an alumina column prepared as follows:

Acidic aluminum oxide (10 kg., M. Woelm) was stirred in a 50 percent aqueous methanol solution. After allowing the mixture to stand, the supernatant solution was decanted and discarded. The alumina was again stirred with 50 vol. percent aqueous methanol and packed into a column having a diameter of 13.5 cm. The alumina column was washed with 50 vol. percent aqueous methanol until a clear effluent was obtained. The column was eluted with 50 vol. percent aqueous methanol at a flow rate of 8—10 ml/minute, collecting fractions having a volume of 240—300 ml. Fractions were monitored by thin-layer bioautography as described in Example 5. On the basis of this data, fractions were combined and yielded purified A-35512 factor B dihydrochloride as follows:

Fractions	Weight
17—21	9.6 g.
22—29	72.0 g.
30—37	117.0 g.

Each of these was crystallized from a concentrated 50 percent aqueous methanol solution at 4°C. A-35512 factor B dihydrochloride thus purified contains about 4.6 percent chlorine. A solution of A-35512 factor B dihydrochloride in 66 vol. percent aqueous dimethylformamide has a pH of about 6.5.

EXAMPLE 7

Preparation of Ionic Chlorine-free A-35512 Factor B

Purified A-35512 factor B dihydrochloride (1 g), obtained as described in Example 6 and dissolved in water (40 ml), was passed through a 2.5-x18-cm. ion-exchange column (Bio-Rad AG3-4x (Registered Trade Mark) in the OH⁻ cycle) at a flow rate of 0.5 ml/minute, eluting with deionized water. The first eluate (5 ml) was discarded. The following eluate (50 ml) was evaporated to dryness under vacuum to give 0.76 g. of ionic chlorine-free A-35512 factor B as a white powder which contained approximately 1.59 percent chlorine. A solution of this ionic chlorine-free A-35512 factor B in 66 percent aqueous dimethylformamide had a pH of 9.13.

EXAMPLE 8

Purification of A-35512 Factor A

Partially purified A-35512 factor A dihydrochloride (1 g), obtained as described in Example 5 (fractions 1—10), was dissolved in 50 vol. % aqueous methanol (5 ml). This solution was chromatographed on a 3-x16-cm acidic alumina (M. Woelm) column, prepared as described in Example 5. The column was eluted with 50 vol. % aqueous methanol at a flow rate of 0.5 ml/minute. The fractions having a volume of 5 ml were collected; all fractions were analyzed by thin-layer bioautography as described in Example 5. On the basis of these tests, fractions 7 through 15 were combined, concentrated under reduced pressure to a small volume and lyophilized to give 0.3 g of A-35512 factor A dihydrochloride (4.71% chlorine).

EXAMPLE 9

Preparation of Ionic Chlorine-free A-35512 Factor A

A-35512 factor A dihydrochloride (200 mg), prepared as described in Example 8, was dissolved in water (10 ml); this solution was passed through a 1.5-x10-cm ion-exchange column (Bio-Rad AG3-4X, in the OH⁻ cycle) at a flow rate of 0.5 ml/minute, eluting with deionized water. The initial eluate (10 ml) was discarded. The following eluate (20 ml) was concentrated to a small volume under reduced pressure and then was lyophilized to give 115 mg of ionic chlorine-free A-35512 factor A.

EXAMPLE 10

Purification of A-35512 Factor C

Partially purified A-35512 factor C dihydrochloride (15 g), obtained as described in Example 5 (fractions 25—31), was dissolved in deionized water (40 ml). This solution was applied to a 4-x115-cm polyamide column (Mackery-Nagel, <0.07 mm, Brinkman Instruments, Inc.; prepared in and washed overnight with water). The column was eluted with deionized water at a flow rate of about 3 ml per minute. The first effluent (250 ml) was discarded; thereafter, fractions having a volume of 25 ml were collected.

Fractions were monitored by thin-layer chromatographic bioautography. Cellulose tlc plates (on aluminum sheets; E. Merck, W. Germany), a sec-butanol:pyridine:acetic acid:water (10:10:3:8) by vol. solvent system, and *Bacillus subtilis* as a detection organism were used. Based on the tlc results, fractions 1 through 33 were combined, concentrated under vacuum to a volume of 150 ml and lyophilized.

Two more lots of partially purified A-35512 factor C dihydrochloride were chromatographed using the same conditions. Each time fractions 1 through 33 were combined, concentrated under vacuum to 150 ml, and lyophilized. The three lyophilized samples from the three columns were combined to give 12.3 grams of partially purified A-35512 factor C dihydrochloride.

This A-35512 factor C dihydrochloride was further purified by chromatography over another polyamide column as above-described, but collecting fractions having a volume of 15 ml, at a flow rate of 1 ml per minute. The column was again monitored by tlc bioautography. Fractions 36 through 58 were combined, concentrated under vacuum to a volume of 150 ml and lyophilized to give 5.3 grams of further-purified A-35512 factor C dihydrochloride.

Final purification of A-35512 factor C dihydrochloride was by chromatography on a 5-x41-cm acidic aluminum oxide (Woelm) column. The column was packed and washed with 50 vol % aqueous methanol. When the washing effluent was clear, the A-35512 factor C (5.3 g dissolved in 30 ml of 50 vol % aqueous methanol) was applied to the column. The column was eluted with 50 vol. % aqueous methanol at a flow rate of one ml per minute. Fractions having a volume of 12 ml were collected and were monitored by tlc bioautography as earlier described. Fractions 22 through 74 were combined, concentrated under vacuum to a volume of 250 ml and lyophilized to give 3.86 grams of A-35512 factor C dihydrochloride.

EXAMPLE 11

Preparation of Ionic Chlorine-free A-35512 Factor C

A-35512 factor C dihydrochloride (200 mg), prepared as described in Example 10, was chromatographed over weakly-basic ion-exchange resin, using the procedure given in Example 9, to give 156 mg of ionic chlorine-free A-35512 factor C. This ionic chlorine-free A-35512 factor C contained approximately 1.90% chlorine.

EXAMPLE 12

Purification A-35512 Factor E

Partially purified A-35512 factor E hydrochloride (8.1 g), obtained as described in Example 5 (fractions 32-44), was dissolved in deionized water (40 ml). This solution was applied to a 5-x100-cm polyamide column (MN, <0.07 mm, Brinkman Instruments, Inc.), prepared in and washed with water overnight. The column was eluted with deionized water at a flow rate of 20 ml per 15 minutes, collecting fractions having a volume of 20 ml. At fraction 118, the eluting solvent was changed to 50 vol. % aqueous methanol. Fractions were monitored by thin-layer chromatographic bioautography as described in Example 10.

Fractions 148 through 195 contained A-35512 factor E. These fractions were combined, concentrated under reduced pressure to a volume of 150 ml, and lyophilized to give 2.7 g of A-35512 factor E hydrochloride.

A portion of this partially purified A-35512 factor E hydrochloride (615 mg) was dissolved in 50 vol. % aqueous methanol (5 ml) and applied to a 1.5-x50-cm acidic aluminum oxide (Woelm) column which had been prepared in and washed with 50 vol. % aqueous methanol until the effluent was clear. The column was eluted with 50 vol. % aqueous methanol at a flow rate of 1 ml per minute, collecting fractions having a volume of 10 ml. Again, fractions were monitored by thin-layer chromatographic bioautography. Fractions 5-8 were combined and concentrated under reduced pressure to a volume of about 10 ml. Deionized water (about 50 ml) was added, and the resulting solution was lyophilized to give 480 mg of A-35512 factor E hydrochloride.

EXAMPLE 13

Preparation of Ionic Chlorine-free A-35512 Factor E

A-35512 Factor E hydrochloride (200 mg), prepared as described in Example 12, was chromatographed over weakly-basic ion-exchange resin, using the procedure given in Example 9, to give 170 mg of ionic chlorine-free A-35512 Factor E. This ionic chlorine-free A-35512 factor E contained approximately 1.72% chlorine.

EXAMPLE 14

Purification of A-35512 Factor H

Partially purified A-35512 factor H hydrochloride (30 g.), obtained as described in Example 5 (fractions 1-10), was dissolved in a minimal amount of methanol:water (7:3) by vol. solution. The resulting solution was adsorbed on an acidic aluminum oxide column (3-x60-cm; Woelm; packed in methanol and eluted with methanol until the effluent was clear). The column was then eluted with

methanol at a flow-rate of four ml/minute. Fractions having a volume of 24 ml were collected. The eluting solvent was changed at fraction 59 to methanol:water (1:1) by vol.

Fractions were monitored by thin-layer chromatography using a chloroform:methanol:ammonium hydroxide (2:3:1) by vol. solvent system and *Bacillus subtilis* bioautography at alkaline pH.

Fractions 51 through 118 were combined and evaporated under vacuum to give 6.4 grams of purified A-35512 factor H hydrochloride.

EXAMPLE 15

Preparation of Ionic Chlorine-free A-35512 Factor H

A-35512 factor H hydrochloride (200 mg), prepared as described in Example 14, was chromatographed over weakly-basic ion-exchange resin, using the procedure given in Example 9, to give 143 mg of ionic chlorine-free A-35512 factor H. This ionic chlorine-free A-35512 factor H contained approximately 1.59% chlorine.

EXAMPLE 16

Preparation of A-35512B Aglycone

A-35512 factor B dihydrochloride (5.0 g), obtained as described in Example 6, was dissolved in water (200 ml). This solution was acidified with 4N hydrochloric acid (14 ml). The resulting solution was refluxed for two hours. The solution then was cooled and evaporated under vacuum to about 3/4 its original volume. Hydrochloric acid (6N) was added dropwise to this solution until precipitation was complete. The resulting precipitate was separated by filtration and dried to give 3.56 g. of crude A-35512B aglycone hydrochloride.

The filtrate was concentrated and analyzed. The filtrate contained glucose, fucose, mannose and rhamnose.

The crude A-35512B aglycone was purified by chromatography on acid-washed alumina (Woelm, Grade I), using a water:methanol (1:9) by vol. solvent system. Elution of the column was monitored by cellulose thin-layer chromatography. The eluted fractions containing A-35512B aglycone were combined and evaporated under vacuum to give 398 mg. of partially-purified product. Comparison thin-layer chromatographic results, using a ninhydrin spray for detection, confirmed that non-bioactive impurities were still present.

A portion of this partially-purified A-35512B aglycone (100 mg) was further purified by chromatography over polyamide (4 g; Machery, Nagel & Co. MN-SC-6, distributed by Brinkmann Instruments Co.; <0.07 mm), eluting with water. This column was also monitored by cellulose thin-layer chromatography as earlier described. The eluted fractions containing A-35512 factor B aglycone were combined and lyophilized to give 64 mg. of purified A-35512 factor B aglycone hydrochloride. (Total yield of 5.08% from starting A-35512 factor B).

EXAMPLE 17

Preparation of A-35512 Factor B Aglycone Free Base

A-35512 factor B aglycone hydrochloride (90 mg), obtained as described in Example 16, was dissolved in 30 ml of methanol-water (1:1) by vol. This solution was neutralized with ion-exchange resin [3.5 ml, Bio-Rad AG-3-4X (OH⁻)]. The resulting solution was stirred for 15 minutes at room temperature. The resin was then removed by filtration. The filtrate was concentrated under vacuum, maintaining the temperature at less than 60°C., to about half volume, and then lyophilized to give 68 mg. of A-35512 factor B aglycone free base.

WHAT WE CLAIM IS:—

1. An antibiotic A-35512 mixture comprising factors A, B, C, E, F, G and H as defined in claims 2—8 respectively.

2. Antibiotic A-35512 factor A which is a white amorphous, basic compound, having an approximate elemental composition of 54.29 percent carbon, 5.19 percent hydrogen, 5.58 percent nitrogen, 33.76 percent oxygen, and 1.69 percent chlorine; and which in its dihydrochloride form is a white amorphous hygroscopic compound which has these characteristics:

a) an approximate elemental composition of 51.03 percent carbon, 5.10 percent hydrogen, 4.75 percent nitrogen, 34.20 percent oxygen, and 4.80 percent chlorine;

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methanol at a flow-rate of four ml/minute. Fractions having a volume of 24 ml were collected. The eluting solvent was changed at fraction 59 to methanol:water (1:1) by vol.

5 Fractions were monitored by thin-layer chromatography using a chloroform:methanol:ammonium hydroxide (2:3:1) by vol. solvent system and *Bacillus subtilis* bioautography at alkaline pH.

Fractions 51 through 118 were combined and evaporated under vacuum to give 6.4 grams of purified A-35512 factor H hydrochloride.

EXAMPLE 15

10 Preparation of Ionic Chlorine-free A-35512 Factor H

A-35512 factor H hydrochloride (200 mg), prepared as described in Example 14, was chromatographed over weakly-basic ion-exchange resin, using the procedure given in Example 9, to give 143 mg of ionic chlorine-free A-35512 factor H. This ionic chlorine-free A-35512 factor H contained approximately 1.59% chlorine.

EXAMPLE 16

Preparation of A-35512B Aglycone

20 A-35512 factor B dihydrochloride (5.0 g), obtained as described in Example 6, was dissolved in water (200 ml). This solution was acidified with 4N hydrochloric acid (14 ml). The resulting solution was refluxed for two hours. The solution then was cooled and evaporated under vacuum to about 3/4 its original volume. Hydrochloric acid (6N) was added dropwise to this solution until precipitation was complete. The resulting precipitate was separated by filtration and dried to give 3.56 g. of crude A-35512B aglycone hydrochloride.

25 The filtrate was concentrated and analyzed. The filtrate contained glucose, fucose, mannose and rhamnose.

30 The crude A-35512B aglycone was purified by chromatography on acid-washed alumina (Woelm, Grade I), using a water:methanol (1:9) by vol. solvent system. Elution of the column was monitored by cellulose thin-layer chromatography. The eluted fractions containing A-35512B aglycone were combined and evaporated under vacuum to give 398 mg. of partially-purified product. Comparison thin-layer chromatographic results, using a ninhydrin spray for detection, confirmed that non-bioactive impurities were still present.

35 A portion of this partially-purified A-35512B aglycone (100 mg) was further purified by chromatography over polyamide (4 g; Machery, Nagel & Co. MN-SC-6, distributed by Brinkmann Instruments Co.; <0.07 mm), eluting with water. This column was also monitored by cellulose thin-layer chromatography as earlier described. The eluted fractions containing A-35512 factor B aglycone were combined and lyophilized to give 64 mg. of purified A-35512 factor B aglycone hydrochloride. (Total yield of 5.08% from starting A-35512 factor B).

EXAMPLE 17

Preparation of A-35512 Factor B Aglycone Free Base

45 A-35512 factor B aglycone hydrochloride (90 mg), obtained as described in Example 16, was dissolved in 30 ml of methanol-water (1:1) by vol. This solution was neutralized with ion-exchange resin [3.5 ml, Bio-Rad AG-3-4X (OH⁻)]. The resulting solution was stirred for 15 minutes at room temperature. The resin was then removed by filtration. The filtrate was concentrated under vacuum, maintaining the temperature at less than 60°C., to about half volume, and then lyophilized to give 68 mg of A-35512 factor B aglycone free base.

50 WHAT WE CLAIM IS:—

1. An antibiotic A-35512 mixture comprising factors A, B, C, E, F, G and H as defined in claims 2—8 respectively.

55 2. Antibiotic A-35512 factor A which is a white amorphous, basic compound, having an approximate elemental composition of 54.29 percent carbon, 5.19 percent hydrogen, 5.58 percent nitrogen, 33.76 percent oxygen, and 1.69 percent chlorine; and which in its dihydrochloride form is a white amorphous hygroscopic compound which has these characteristics:

60 a) an approximate elemental composition of 51.03 percent carbon, 5.10 percent hydrogen, 4.75 percent nitrogen, 34.20 percent oxygen, and 4.80 percent chlorine;



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- b) an infrared absorption spectrum in KBr pellet with significant absorption maxima at the following frequencies (cm^{-1}): 3405 (strong), 3300 (shoulder), 2950 (weak), 1750 (weak), 1670 (strong), 1625 (shoulder), 1602 (strong), 1520 (strong), 1470 (weak), 1440 (weak), 1405 (weak), 1345 (shoulder), 1312 (medium), 1225 (medium), 1180 (weak), 1130 (weak), 1080 (strong), and 1020 (shoulder);
- c) ultraviolet absorption spectra, in acidic and neutral methanol, with an absorption maximum at 282 nm (ϵ 11,700) and, in basic methanol, an absorption maximum at 292 nm (ϵ 14,000), calculated using a molecule weight of 2,000, and with end absorption at 225 nm;
- d) a molecular weight of about 2106, as determined by titration;
- e) four electrometrically titrable groups in 66 vol. % aqueous dimethylformamide with pK_a values of approximately 7.35, 9.09, 10.49, and 12.44 (initial pH 6.2);
- f) the following specific rotations:
- $[\alpha]_D^{25} - 100^\circ$ (c 1, H_2O)
- $[\alpha]_{365}^{25} - 400^\circ$ (c 1, H_2O);
- g) is soluble in water, partially soluble in methanol and ethanol, and insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane;
- h) an amino-acid analysis showing the presence of at least five amino acid residues, one of which is glycine;
- i) an R_f value of approximately 0.21 on paper chromatography in a 1-butanol:pyridine:acetic acid:water (15:10:3:12) by vol. solvent system, using *Sarcina lutea* as the detection organism;
- j) a ^{13}C nuclear magnetic resonance spectrum in D_2O with the following characteristics:

No.	PPM	Height
3	175.3	0.8
4	173.0	2.1
5	172.1	2.0
6	171.4	1.5
7	170.9	2.7
8	170.5	2.3
9	169.5	1.6
10	159.0	1.3
11	157.9	2.3
12	156.2	2.6
13	155.6	2.3
14	155.3	2.4
15	154.4	1.1
16	136.3	1.4
17	136.0	1.0
18	135.1	1.4
19	133.5	1.2
20	129.6	1.6
21	129.1	1.7
22	128.7	1.8
23	127.5	1.0
24	126.0	1.4
25	124.3	2.8
26	122.1	1.6
27	109.9	1.3
28	107.4	1.6
29	101.7	0.9
30	77.6	3.8
31	76.3	4.6
32	75.5	2.6
33	74.8	2.5
34	74.5	2.4
35	73.4	3.7
36	72.8	6.0
37	72.0	4.4

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No.	PPM	Height
38	70.9	7.0
39	69.6	2.8
41	65.4	1.7
42	61.7	3.1
43	56.7	1.7
44	55.5	1.3
45	54.7	0.8
46	24.6	0.9
47	19.1	1.7
48	17.9	2.0
49	17.2	1.9
50	16.7	3.2
51	16.2	3.0

k) is stable for 72 hours in aqueous solutions having a pH of from 3 to 10; and the pharmaceutically-acceptable acid addition salts of A-35512 factor A.

3. Antibiotic A-35512 factor B which is a white amorphous, basic compound having:

a) an approximate elemental composition of 53.97 percent carbon, 4.75 percent hydrogen, 5.25 percent nitrogen, 34.29 percent oxygen, and 1.59 percent chlorine;

b) ultraviolet absorption spectra, in acidic and neutral methanol, with an absorption maximum at 282 nm (ϵ 15000) and, in basic methanol, an absorption maximum at 292 nm (ϵ 16000) calculated using a molecular weight of 2,000;

c) a molecular weight of about 2143, as determined by titration;

d) four electrometrically titratable groups in 66 vol. % aqueous dimethylformamide with pK_a values of approximately 7.15, 8.81, 10.20, and 12.00;

e) the following specific rotations:

$$[\alpha]_D^{25} - 123^\circ \text{ (c 1, H}_2\text{O)}$$

$$[\alpha]_{365}^{25} - 446^\circ \text{ (c 1, H}_2\text{O)};$$

f) an empirical formula in the range of $C_{97-99}H_{101-106}N_{2-3}O_{48-48}Cl$; and which in its dihydrochloride form is a white, hygroscopic, crystalline compound (from 50 vol. percent aqueous methanol) which has these characteristics:

a') an approximate elemental composition of 52.57 percent carbon, 4.80 percent hydrogen, 5.66 percent nitrogen, 32.86 percent oxygen, and 4.51 percent chlorine;

b') an infrared absorption spectrum in KBr pellet with significant absorption maxima at the following frequencies (cm^{-1}): 3420 (strong), 3300 (shoulder), 2950 (weak), 1752 (weak), 1675 (strong), 1630 (shoulder), 1605 (strong), 1520 (strong), 1470 (weak), 1440 (weak), 1410 (weak), 1345 (shoulder), 1312 (medium), 1225 (medium), 1180 (weak), 1135 (weak), 1080 (strong), and 1020 (weak);

c') ultraviolet absorption spectra, in acidic and neutral methanol, with an absorption maximum at 282 nm (ϵ 12,000) and, in basic methanol, an absorption maximum at 292 nm (ϵ 14,000), calculated using a molecular weight of 2,000, and with end absorption at 225 nm;

d') a molecular weight of about 2027, as determined by titration;

e') four electrometrically titratable groups in 66 vol. % aqueous dimethylformamide with pK_a values of approximately 7.15, 8.87, 10.30, and 12.10;

f') the following specific rotations:

$$[\alpha]_D^{25} - 128^\circ \text{ (c 1, H}_2\text{O)}$$

$$[\alpha]_{365}^{25} - 475^\circ \text{ (c 1, H}_2\text{O)};$$

g') is soluble in water, partially soluble in methanol and ethanol, and insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane;

h') an amino-acid analysis showing the presence of at least five amino acid residues, one of which is glycine;



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i') an R_f value of approximately 0.34 on paper chromatography in a 1-butanol:pyridine:acetic acid:water (15:10:3:12) by vol. solvent system, using *Sarcina lutea* as the detection organism;

j') a ^{13}C nuclear magnetic resonance spectrum in D_2O with the following characteristics:

5	No.	PPM	Height	5
	2	173.0	4.1	
	3	171.9	3.7	
	4	171.6	3.3	
10	5	171.0	5.8	10
	6	170.8	5.0	
	7	169.6	3.6	
	8	159.0	4.1	
	9	157.9	4.4	
15	10	157.5	3.7	15
	11	156.6	4.8	
	12	155.6	6.1	
	13	155.3	4.2	
	14	154.9	3.3	
20	15	154.3	4.2	20
	16	151.7	3.3	
	17	144.3	3.1	
	18	136.7	3.5	
	19	136.2	4.9	
25	20	135.4	4.0	25
	21	135.2	4.4	
	22	133.6	4.2	
	23	133.3	4.1	
	24	129.8	1.7	
30	25	129.3	3.0	30
	26	128.8	2.6	
	27	127.6	1.5	
	28	126.1	3.9	
	29	124.2	5.6	
35	30	122.4	1.4	35
	31	122.0	4.4	
	32	120.7	3.3	
	33	116.5	2.7	
	34	109.5	0.8	
40	35	108.2	1.1	40
	36	107.7	2.7	
	37	104.5	1.7	
	38	101.8	2.9	
	39	100.9	1.6	
45	40	98.2	1.0	45
	41	76.9	1.2	
	42	76.1	1.8	
	43	74.1	2.0	
	44	73.5	2.7	
50	45	72.7	2.4	50
	46	72.3	4.0	
	47	71.0	7.1	
	48	70.3	2.5	
	49	69.7	2.5	
55	51	64.6	1.2	55
	52	62.0	1.5	
	53	58.0	1.3	
	54	56.8	1.7	
	55	55.4	3.9	
60	56	54.3	2.5	60
	57	24.5	2.0	
	58	17.9	3.0	
	59	17.2	2.0	
	60	16.3	2.5	

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k') is stable for up to 72 hours in aqueous solutions having a pH of from 3 to 10;
l') an X-ray powder diffraction pattern (Cu⁺⁺ radiation, 1.5405 Å, nickel filter)
having the following interplanar spacings in angstroms (d):

	d	Relative Intensity	
5	17.15	100	5
	12.90	80	
	10.85	70	
	9.25	70	
10	8.87	60	10
	8.22	50	
	7.86	50	
	6.93	40	
	6.20	40	
15	5.62	40	15
	5.04	05	
	4.02	02	
	3.54	02	

m') contains the following sugars: glucose, fucose, mannose, rhamnose, and
3 - amino - 2,3,6 - trideoxy - 3 - C - methyl - L - xylo - hexopyranose;
n') has at least one hydroxyl group capable of esterification; and the
pharmaceutically-acceptable acid addition salts of A-35512 factor B.

4. Antibiotic A-35512 factor C which is a white, amorphous, basic compound
having:

- 25 a) an approximate elemental composition of 53.93 percent carbon, 5.15 percent hydrogen, 5.80 percent nitrogen, 32.35 percent oxygen, and 1.90 percent chlorine; 25
- b) a molecular weight of approximately 1862; and which in its dihydrochloride form is a white amorphous compound which has these characteristics:
- 30 a') an approximate elemental composition of 51.76 percent carbon, 5.07 percent hydrogen, 5.61 percent nitrogen, 30.29 percent oxygen, and 4.88 percent chlorine; 30
- b') an infrared absorption spectrum in KBr pellet with significant absorption maxima at the following frequencies (cm⁻¹): 3370 (strong), 3280 (shoulder), 3040 (shoulder), 2980 (shoulder), 2920 (weak), 1740 (weak), 1658 (strong), 1620 (weak), 1589 (medium), 1503 (strong), 1460 (weak), 1428 (medium), 1385 (weak), 1330 (weak), 1295 (medium), 1210 (strong), 1162 (medium), 1120 (weak), 1060 (strong), and 1005 (medium); 35
- c') ultraviolet absorption spectra with an absorption maximum, in acidic and neutral methanol, a 282 nm (ε 14,600) and, in basic methanol, at 292 nm (ε 16,400) calculated using a molecular weight of 2,000; 40
- d') a molecular weight of about 1982, as determined by titration;
- e') three electrometrically titratable groups in 66% vol. aqueous dimethylformamide with pK_a values of approximately 7.30, 8.92, and 10.99; 45
- f') the following specific rotations: 45

$$[\alpha]_D^{25} - 161^\circ \text{ (c 1.05, H}_2\text{O)}$$

$$[\alpha]_{365}^{25} - 614^\circ \text{ (c 1.05, H}_2\text{O)};$$

- 50 g') is soluble in water, dimethyl sulfoxide, and aqueous dimethylformamide, partially soluble in methanol and ethanol, and insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile, and dioxane; 50
- h') an amino-acid analysis showing the presence of five amino-acid residues;
- 55 i') an R_f value of approximately 0.46 on paper chromatography in a 1-butanol:pyridine:acetic acid:water (15:10:3:12) by vol. solvent system, using *Sarcina lutea* as the detection organism; 55
- j') a ¹³C nuclear magnetic resonance spectrum in D₂O with the following characteristics:



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	No.	PPM	Height	
	1	172.9	2.5	
	2	172.2	2.0	
	3	171.5	2.2	
5	4	171.0	3.9	5
	5	169.6	2.0	
	6	158.6	1.8	
	7	157.8	3.0	
	8	156.5	2.1	
10	9	156.1	2.8	10
	10	155.6	4.2	
	11	154.6	3.9	
	12	151.1	1.5	
	13	143.3	1.4	
15	14	136.0	3.2	15
	15	135.4	2.7	
	16	133.2	3.7	
	17	128.7	2.3	
	18	126.5	3.1	
20	19	124.6	2.1	20
	20	123.9	2.7	
	21	121.5	2.2	
	22	120.1	1.2	
	23	118.0	1.7	
25	24	116.5	1.2	25
	25	107.8	2.7	
	26	104.5	1.9	
	27	101.7	1.9	
	28	94.5	1.0	
30	29	75.9	3.0	30
	30	74.3	2.0	
	31	73.4	2.3	
	32	72.1	3.5	
	33	70.9	4.3	
35	34	68.7	2.9	35
	36	64.3	1.3	
	37	62.2	1.6	
	38	56.1	1.4	
	39	55.2	3.5	
40	40	54.2	2.1	40
	41	24.3	1.9	
	42	17.9	2.2	
	43	17.1	2.0	
	44	16.2	2.0	

45 k') is stable in aqueous solutions having a pH of from 3 to 10, for up to 146 hours; and the pharmaceutically-acceptable acid addition salts of A-35512 factor C. 45

50 5. Antibiotic A-35512 factor E which is a white, amorphous, basic compound having an approximate elemental composition of 54.84 percent carbon, 4.73 percent hydrogen, 5.26 percent nitrogen, 32.67 percent oxygen, and 1.72 percent chlorine; and which in its hydrochloride form is a white amorphous compound which has these characteristics: 50

55 a) an approximate elemental composition of 52.67 percent carbon, 4.59 percent hydrogen; 5.55 percent nitrogen, 33.51 percent oxygen, and 3.62 percent chlorine; 55

b) an infrared absorption spectrum in KBr pellet with significant absorption maxima at the following frequencies (cm⁻¹): 3360 (strong), 3220 (shoulder), 2900 (weak), 1725 (weak), 1650 (strong), 1580 (medium), 1498 (strong), 1450 (weak), 1419 (weak), 1295 (medium), 1205 (medium), 1172 (medium), 1110 (weak), 1060 (strong), and 1000 (weak); 60

c) ultraviolet absorption spectra with the following absorption maxima: in neutral methanol, 270 nm (sh) and 359 nm (ϵ 16,216); in acidic methanol, 286 nm (ϵ 18,018) and 310 nm (sh); in basic methanol, 270 nm (sh), 300 nm (ϵ 16,216), and 354 nm (ϵ 17,568) calculated using a molecular weight of 2,000; 60

d) a molecular weight of about 2018, as determined by titration;
 e) three electrometrically titratable groups in 66 vol. % aqueous dimethylformamide with pK_a values of approximately 6.30, 9.09, and 11.62 (initial pH 5.45);

f) the following specific rotation:

$$[\alpha]_D^{25} - 108.3^\circ \text{ (c 1, H}_2\text{O)};$$

g) is soluble in water, partially soluble in methanol and ethanol, and insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane;

h) an amino acid analysis showing the presence of six amino acid residues;

i) an R_f value of approximately 0.64 on paper chromatography in a 1-butanol:pyridine:acetic acid:water (15:10:3:12) by vol. solvent system, using *Sarcina lutea* as the detection organism; and the pharmaceutically-acceptable acid-addition salts of A-35512 factor E.

6) Antibiotic A-35512 factor F having an R_f value of approximately 0.81 in a paper-chromatographic system, using *Sarcina lutea* as the detection organism in a 1-butanol:pyridine:acetic acid:water (15:10:3:12) by vol. solvent system.

7. Antibiotic A-35512 factor G having an R_f value of approximately 0.93 in a paper-chromatographic system using *Sarcina lutea* as the detection organism in a 1-butanol:pyridine:acetic acid:water (15:10:3:12) by vol. solvent system.

8. Antibiotic A-35512 factor H which is a white, amorphous, basic compound having:

a) an approximate elemental composition of 53.76 percent carbon, 5.32 percent hydrogen, 5.53 percent nitrogen, 33.48 percent oxygen, and 1.59 percent chlorine;

b) an empirical formula in the range of $C_{85-87}H_{103-107}N_5O_{38-40}Cl$;

c) a molecular weight of approximately 1908, based on elemental composition; and which in its hydrochloride form is a white amorphous, hygroscopic compound which has these characteristics:

a') an approximate elemental composition of 53.10 percent carbon, 5.37 percent hydrogen, 5.35 percent nitrogen, 30.12 percent oxygen, and 3.78 percent chlorine;

b') an infrared absorption spectrum in KBr pellet with significant absorption maxima at the following frequencies (cm^{-1}): 3410 (strong), 3240 (shoulder), 2940 (weak), 1670 (strong), 1630 (shoulder), 1605 (strong), 1520 (strong), 1470 (weak), 1442 (weak), 1400 (weak), 1345 (shoulder), 1310 (medium), 1225 (medium), 1180 (weak), 1135 (weak), 1080 (strong), and 1020 (shoulder).

c') ultraviolet absorption spectra which show, in acidic and neutral methanol, an absorption maximum at 282 nm (ϵ 12,500) and, in basic methanol, an absorption maximum at 292 nm (ϵ 14,000) calculated using a molecular weight of 2,000, and end absorption at 225 nm;

d') five electrometrically titratable groups in 66 vol. % aqueous dimethylformamide with pK_a values of approximately 5.0, 7.46, 9.80, 11.43 and 13.02 (neutral pH 5.93);

e') the following specific rotation:

$$[\alpha]_D^{25} - 123.5^\circ \text{ (c 1, H}_2\text{O)};$$

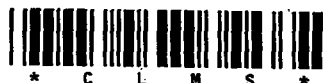
f') is soluble in water, partially soluble in methanol and ethanol, and insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane;

g') an amino acid analysis showing the presence of five amino acid residues, one of which is glycine;

h') approximate R_f values in the following paper-chromatographic systems, using *Sarcina lutea* as the detection organism:

Solvent System	R_f Value
1-butanol:pyridine:acetic acid:water (15:10:3:12) by vol.	0.15
CH_3OH :0.1N HCl (3:1) by vol.	0.47
1-propanol: NH_4OH : H_2O (6:3:1) by vol.	0.11

i') a ^{13}C nuclear-magnetic-resonance spectrum in D_2O with the following characteristics:



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	No.	PPM	Height	
	2	177.2	2.7	
	3	171.6	5.2	
	4	170.9	5.8	
5	5	169.6	4.7	5
	6	158.9	3.1	
	7	157.6	4.3	
	8	156.6	3.8	
	9	155.6	4.1	
10	10	155.4	3.8	10
	11	154.3	2.4	
	12	151.3	1.6	
	13	137.7	2.0	
	14	136.7	2.2	
15	15	136.0	4.0	15
	16	135.3	1.9	
	17	133.5	5.0	
	18	129.4	3.7	
	19	127.3	1.3	
20	20	126.1	3.2	20
	21	124.2	6.9	
	22	122.6	4.1	
	23	107.6	2.7	
	24	101.8	1.8	
25	25	76.2	2.8	25
	26	73.5	4.4	
	27	72.3	7.4	
	28	71.0	12.2	
	29	69.7	4.6	
30	31	61.6	3.5	30
	32	56.8	1.8	
	33	55.4	2.8	
	34	55.0	1.5	
	35	24.5	2.6	
35	36	17.9	4.6	35
	37	17.2	2.6	
	38	16.3	3.6	

j') is stable for up to 72 hours in aqueous solutions having a pH of from 3 to 10; and the pharmaceutically-acceptable acid-addition salts of A-35512 factor H.

9. A-35512 factor B aglycone, which in its hydrochloride form is a white amorphous compound having these characteristics:

a) an approximate elemental composition of 54.29 percent carbon, 4.34 percent hydrogen, 7.40 percent nitrogen, 28.95 percent oxygen, and 5.02 percent chlorine;

b) an infrared absorption spectrum in Nujol mull with significant absorption maxima at the following frequencies (cm^{-1}): 3440 (shoulder), 3340 (shoulder), 3215 (strong), 2950 (shoulder), 2910 (strong), 2840 (strong), 2640 (shoulder), 1735 (weak), 1655 (strong), 1590 (medium), 1500 (strong), 1460 (strong), 1378 (medium), 1365 (shoulder), 1298 (medium), 1215 (medium), 1155 (medium), 1120 (shoulder), 1105 (weak), 1060 (weak), 1040 (weak), 1008 (medium), 925 (weak), 875 (weak), 765 (shoulder), and 718 (weak);

c) ultraviolet absorption spectra, in acidic and neutral methanol, with an absorption maximum at 282 nm ($E_{1\%}^{1\text{cm}}$ 102.62) and, in basic methanol, an absorption maximum at 302 nm ($E_{1\%}^{1\text{cm}}$ 182.09);

d) a molecular weight of about 1282, as determined by titration;

e) three electrometrically titratable groups in 66 vol. % aqueous dimethylformamide with pK_a values of approximately 7.5, 9.25, and 11.0;

f) the following specific rotations:

$[\alpha]_D^{25} - 178^\circ$ (c 5, CH_3OH)

$[\alpha]_{365}^{25} - 716.8^\circ$ (c 5, CH_3OH);

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g) is soluble in water and methanol, but is insoluble in benzene, chloroform, acetone, diethyl ether, ethyl acetate, toluene, n-hexane, acetonitrile and dioxane;
 h) an amino acid analysis showing the presence of at least four amino acid residues one of which is glycine;
 i) an R_f value of approximately 0.80 on cellulose thin-layer chromatography (aluminum support) in a 1-butanol:pyridine:acetic acid:water (15:10:3:12) by vol. solvent system, using *Sarcina lutea* as the detection organism;
 j) an R_f value of approximately 0.26 on silica gel thin-layer chromatography, in a methanol:chloroform:conc. NH_4OH (3:2:1) by vol. solvent system;
 k) a ^{13}C nuclear magnetic resonance spectrum in $\text{DMSO}-d_6$ at 90°C . with the following characteristics:

	No.	PPM	Height	
	1	187.8	37.2	
	2	172.0	40.9	
15	3	170.7	45.2	15
	4	170.1	46.9	
	5	169.6	47.7	
	6	168.4	57.6	
	7	166.7	52.5	
20	8	157.4	49.9	20
	9	156.6	45.5	
	10	155.7	55.8	
	11	155.6	71.5	
	12	155.4	56.7	
25	13	154.5	50.5	25
	14	149.3	43.0	
	15	138.8	38.8	
	16	136.9	54.3	
	17	136.3	40.2	
30	18	135.2	31.7	30
	19	134.7	28.4	
	20	133.8	40.9	
	21	128.2	102.9	
	22	126.2	77.2	
35	23	123.0	57.5	35
	24	121.3	38.1	
	25	117.7	44.4	
	26	117.0	31.5	
	27	108.6	31.0	
40	28	106.7	47.9	40
	29	105.7	81.2	
	30	103.2	26.5	
	31	93.6	33.4	
	32	74.9	39.8	
45	33	71.8	33.9	45
	34	68.9	40.2	
	35	63.2	43.1	
	36	60.4	33.6	
	37	57.1	57.8	
50	38	55.2	33.1	50
	39	53.2	30.1	
	40	51.8	36.0	
	44	23.8	55.7	
	45	17.1	47.8	
55	46	0.0	43.7	55

l) contains 3 - amino - 2,3,6 - trideoxy - 3 - C - methyl - L - xylo - hexopyranose moiety;
 m) has at least one hydroxyl group capable of esterification; and which in its free-base form is a white amorphous compound having these characteristics:
 a') an approximate elemental composition of 52.65% carbon, 4.57% hydrogen, 6.91% nitrogen, 27.04% oxygen, and 2.94% chlorine;



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- 5 b') an infrared absorption spectrum in KBr pellet with significant absorption maxima, at the following frequencies (cm^{-1}): 3360 (strong), 3260 (shoulder), 2940 (shoulder), 1735 (shoulder), 1660 (strong), 1598 (medium), 1510 (strong), 1440 (medium), 1295 (weak), 1215 (medium), 1165 (medium), 1122 (weak), 1070 (weak), 1018 (strong), 940 (weak), and 920 (weak);
- 10 c') ultraviolet absorption spectra with an absorption maximum, in acidic and neutral methanol, at 282 nm ($E_{1\%}^{1\text{cm}}$ 43.65) and, in basic methanol, at 301 nm ($E_{1\%}^{1\text{cm}}$ 67.46);
- 10 d') five electrometrically titratable groups in 66 vol. % aqueous dimethylformamide with pK_a values of approximately 6.2, 8.2, 10.1, 11.4, and 12.4;
- e') the following specific rotation:

$[\alpha]_D^{25} - 64.5^\circ$ (c 3, DMSO);

15 and the pharmaceutically-acceptable acid addition salts of A-35512 factor B aglycone. 15

10. A process for producing antibiotic A-35512 mixture comprising factors A, B, C, E, F, G and H; A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G and A-35512 factor H; and the aglycone derivative of factor B as defined in claims 1—9 respectively comprising: 20

a) cultivating *Streptomyces candidus* NRRL 8156 or an A-35512 mixture producing mutant thereof in a culture medium containing assimilable sources of carbohydrate, nitrogen, and inorganic salts under submerged aerobic fermentation conditions; 25

b) optionally, separating antibiotic A-35512 mixture from the culture medium; c) optionally, isolating antibiotic A-35512 factors A, B, C, E, F, G and H from the antibiotic A-35512 mixture; and 25

d) optionally, preparing antibiotic A-35512 factor B aglycone from antibiotic A-35512 factor B. 30

11. A feed composition suitable for the nutrition of ruminant animals having a developed rumen function comprising a feed and propionate-increasing concentration of a member selected from antibiotic A-35512 mixture, A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G, A-35512 factor H and A-35512 factor B aglycone as defined in claims 1—9 respectively, and the pharmaceutically-acceptable acid addition salts of A-35512 factor A, B, C, E, F, G and H, and of A-35512 factor B aglycone. 35

12. A feed composition suitable for the nutrition of poultry comprising a feed and a feed-efficiency increasing concentration of a member selected from antibiotic A-35512 mixture, A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G, A-35512 factor H and A-35512 factor B aglycone as defined in claims 1—9 respectively, and the pharmaceutically-acceptable acid addition salts of A-35512 factors A, B, C, E, F, G and H, and of A-35512 factor B aglycone. 40

13. A method of increasing feed-utilization efficiency in ruminant animals having a developed rumen function comprising orally administering to said animals an effective propionate-increasing amount of a member selected from antibiotic A-35512 mixture, A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G, A-35512 factor H and A-35512 factor B aglycone, as defined in claims 1—9 respectively, and the pharmaceutically-acceptable acid addition salts of A-35512 factors A, B, C, E, F, G and H, and of A-35512 factor B aglycone. 45

14. A method of increasing feed-utilization efficiency in poultry comprising orally administering to said animals an effective amount of a member selected from antibiotic A-35512 mixture, A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G, A-35512 factor H and A-35512 factor B aglycone as defined in claims 1—9 respectively, and the pharmaceutically-acceptable acid addition salts of A-35512 factors A, B, C, E, F, G and H, and of A-35512 factor B aglycone. 50

15. The antibiotic A-35512 mixture comprising factors A, B, C, E, F, G and H; A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G and A-35512 factor H; and the aglycone derivative of factor B, as defined in claims 1—9 respectively, and the pharmaceutically acceptable acid addition salts of factors A, B, C, E and H and of the aglycone 55 60

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derivative of factor B substantially as hereinbefore described with particular reference to any one of the examples.

5 16. A process for producing antibiotic A-35512 mixture comprising factors A, B, C, E, F, G and H; A-35512 factor A, A-35512 factor B, A-35512 factor C, A-35512 factor E, A-35512 factor F, A-35512 factor G and A-35512 factor H; and of the aglycone derivative of factor B, as defined in claims 1—9 respectively, substantially as hereinbefore described with particular reference to any one of the examples.

10 17. A feed composition suitable for the nutrition of ruminant animals having a developed rumen function as claimed in claim 11 substantially as hereinbefore described.

18. A feed composition suitable for the nutrition of poultry as claimed in claim 12 substantially as hereinbefore described.

15 19. A method of increasing feed-utilization efficiency in ruminant animals having a developed rumen function as claimed in claim 13 substantially as hereinbefore described.

20 20. A method of increasing feed-utilization efficiency in poultry as claimed in claim 14 substantially as hereinbefore described.

21. *Streptomyces candidus* NRRL 8156 in a synthetic cultive medium containing assimilable sources of carbohydrate, nitrogen and inorganic salts.

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