NOVEL AVERMECTINS PRODUCED BY MUTATIONAL BIOSYNTHESIS

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Avermectins with a wide range of novel C-25 substituents have been prepared by feeding carboxylic acids or their biosynthetic precursors to a *Streptomyces avermitilis* mutant strain ATCC 53568. This organism lacks the ability to form isobutyric and *S*-2-methylbutyric acids from their 2-oxo acid precursors and thus is unable to produce natural avermectins unless supplied with these acids. The novel avermectins produced by mutational biosynthesis possess broad-spectrum antiparasitic activity.

The avermectins are a group of antiparasitic macrolides[1] produced by *Streptomyces avermitilis*. Their biosynthesis has been elucidated by studies on the incorporation of labelled precursors[2,3]. An unusual feature of the biosynthetic pathway is the role of isoleucine and valine as precursors for the C-25 substituents of the "a" and "b" series avermectins, respectively. It is likely that the incorporation of these amino acids involves conversion to the coenzyme A derivatives of the branched-chain carboxylic acids *S*-2-methylbutyric acid and isobutyric acid[4]. The preceding paper presents evidence supporting this hypothesis and describes the isolation and properties of a mutant of *S. avermitilis* which lacks functional branched-chain 2-oxo acid dehydrogenase activity[5]. The availability of this blocked mutant presented the attractive possibility that alternative carboxylic acids, fed to a suitable fermentation of this organism, might be incorporated to produce novel avermectins. The application of such a technique to the production of novel antibiotics has been termed "mutational biosynthesis"[6]. In this paper we describe the use of such a process to prepare avermectins modified at the C-25 position. We have previously described some of these results in a patent application[7] and recently our results have been corroborated by another group[8]. This is the first time, to our knowledge, that novel macrolide antibiotics have been produced by feeding analogues of early biosynthetic intermediates to a blocked mutant. In other cases late-stage analogues have been fed to organisms after the macrolide ring has been formed[6].

We further describe an HPLC assay to detect the production of novel avermectins and their isolation, purification, characterization and biological properties.

Experimental

Organism

S. avermitilis mutant strain ATCC 53568, a spontaneous, morphologically-stable mutant of the original

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Fig. 1. Structures of the novel avermectins.

Avermectin	R_1	R_2	X-Y
A1	CH_3	As listed in	CH=CH
A2	CH_3	Table 2	CH_2-CHOH
B1	H		CH=CH
B2	H		CH_2-CHOH

branched-chain 2-oxo acid dehydrogenase-deficient mutant (ATCC 53567)[5], was used throughout this study. The organisms was stored at $-70°C$ as whole broth prepared in a seed medium consisting of starch (Nadex, Laing National) 20 g, Pharmamedia (Traders Protein) 15 g, Ardamine pH (Yeast Products Inc.) 5 g and calcium carbonate 1 g in 1 litre of tap water at pH 7.2.

Flask Fermentation

A thawed suspension of the above organism, 2 ml, was used to inoculate a flask containing 50 ml of the same medium. After 48 hours incubation at 28°C on a rotary shaker at 180 rpm, 2 ml of the broth was used to inoculate a flask containing 50 ml of a production medium consisting of starch (Nadex, Laing National) 80 g, calcium carbonate 7 g, Pharmamedia (Traders Protein) 5 g, dipotassium hydrogen phosphate 1 g, magnesium sulfate 1 g, glutamic acid 0.6 g, ferrous sulfate heptahydrate 0.01 g, zinc sulfate 0.001 g and manganous sulfate 0.001 g in 1 litre of tap water. The carboxylic acid or precursor was dissolved in methanol and added to the fermentation 24 hours after inoculation to give a final concentration of 0.2 g/litre. The fermentation was incubated for a total of 14 days at 28°C then the broth was centrifuged (2,500 rpm for 2 minutes) and the supernatant discarded. The mycelial pellet was extracted with acetone (15 ml) followed by dichloromethane (30 ml) and the organic phase separated, filtered then evaporated to dryness. The residue was taken up in methanol (1 ml) ready for HPLC assay.

HPLC Assay

The assays were performed on a Hewlett-Packard 1090A liquid chromatograph equipped with a scanning diode-array detector. The latter facilitated peak identification due to the characteristic UV chromophore of the avermectins. The column used was a Beckman Ultrasphere C-18, 5 μm, (4.6 mm × 25 cm) maintained at 40°C. Elution was performed with a linear gradient of methanol - water from 80 : 20 to 95 : 5 over 40 minutes at 0.85 ml/minute (25 μl of the above methanolic solution was injected on to the column).

Materials

Unless otherwise indicated, all the carboxylic acids and precursors were obtained from Aldrich, except for the following; 2,3-dimethylbutyric acid and thiophene-2-propionic acid were purchased from Kodak, 2-methylpent-4-enoic acid and ethylthioacetic acid from Lancaster Synthesis and methylthioacetic acid

Table 1. HPLC Rt's of novel avermectins.

Carboxylic acid or precursor	HPLC Rt (minutes)			
	B2	A2	B1	A1
Cyclopropane carboxylic acid	8.3	10.6	15.0	19.3
Cyclobutane carboxylic acid	11.7	14.3	19.9	23.4
Cyclopentane carboxylic acid	14.2	17.4	23.0	26.7
Cyclohexane carboxylic acid	16.9	20.3	26.0	29.6
Cycloheptane carboxylic acid	18.6	22.6	29.0	33.3
2-Thienoic acid	8.5	11.3	15.9	19.9
3-Thienoic acid	8.8	11.1	16.1	19.3
2-Furoic acid	7.6	9.5	14.6	16.5
3-Furoic acid	7.6	9.5	14.6	16.5
5-Methylthiophene-2-carboxylic acid	11.8	14.4	19.9	23.5
Thietane-3-carboxylic acid[17]	8.3	10.6	14.9	17.8
2-Tetrahydrofuroic acid	7.5	9.7	13.8	16.8
3-Tetrahydrothiophene carboxylic acid[18]	9.2	11.8	16.5	19.9
4-Tetrahydropyran carboxylic acid[9]	8.1	10.4	14.5	16.3
4-Tetrahydrothiopyran carboxylic acid[19]	10.8	12.9	18.3	20.8
3,4-Dihydropyran-2-carboxylic acid	10.3	12.0	16.8	20.7
1-Cyclopentene carboxylic acid	12.5	15.3	21.1	24.7
3-Cyclopentene carboxylic acid[20]	11.8	14.2	20.9	24.5
1-Cyclohexene carboxylic acid	14.7	17.8	24.1	27.7
2-Methylcyclopropane carboxylic acid	11.6	12.3	19.5	22.1
3-Methylcyclobutane carboxylic acid[21]	13.6	16.9	22.2	25.3
3-Methylcyclohexane carboxylic acid[22]	18.4	22.9	28.7	33.0
4-Methylcyclohexane carboxylic acid[23]	20.1	23.6	28.9	33.2
4-Exomethylenecylohexane carboxylic acid[24]	15.5	19.4	25.4	29.0
2,3-Dimethylbutyric acid	14.9	17.1	24.0	27.4
2-Methylpentanoic acid	14.5	17.7	24.9	28.5
2-Methylhexanoic acid	16.8	20.0	27.6	31.1
2-Ethylbutyric acid	15.2	18.1	24.6	28.3
Methylthiolactic acid[25]	9.1	11.4	15.1	18.4
2-Methyl-4-methoxy butyric acid[26]	10.0	12.3	16.5	20.0
2-Methylpent-4-enoic acid	12.9	15.8	20.9	24.8
2-Methylpent-2-enoic acid	12.8	15.4	20.5	24.6
Methacrylic acid	9.6	12.4	16.7	19.7
Tiglic acid	11.1	13.6	19.2	22.8
2-Methylpent-4-ynoic acid	8.9	11.5	16.2	19.5
Cyclobutane methanol	11.7	14.3	19.9	23.4
Aminomethyl cyclobutane	11.7	14.3	19.9	23.4
Ethyl cyclobutane carboxylate	11.7	14.3	19.9	23.4
Cyclopentane methanol	14.2	17.4	23.0	26.7
Cyclopentane propanol	14.2	17.4	23.0	26.7
Cyclopentane propionic acid	14.2	17.4	23.0	26.7
Cyclohexane carboxamide	16.9	20.3	26.0	29.6
Cyclohexane propionic acid	16.9	20.3	26.0	29.6
Thiophene-2-propionic acid	8.5	11.3	15.9	19.9

from Fluka.

Isolation

In order to confirm the structural assignments listed in Table 1, and to investigate the biological properties of the novel avermectins, the flask fermentations were scaled-up and the products isolated as described below.

Large Scale Fermentation

A frozen inoculum (2 ml) of a culture of *S. avermitilis* ATCC 53568 was transferred to two 300-ml

Erlenmeyer flasks, each containing 50 ml of the seed medium described above, and incubated at 28°C on a rotary shaker with a 2.5-cm throw at 170 rpm for 2 days. The resultant vegetative growth was used to inoculate two 3-litre Fernbach conical flasks, each containing 700 ml of the seed medium. These flasks were incubated under the same conditions as above and after 2 days the total contents of both flasks were transferred to a 100-litre mechanically agitated vessel containing 70 litres of the seed medium and incubated at 28°C for a further 2 days. This third stage seed culture was then inoculated into a 2,000-litre fermenter containing 1,200 litres of the production medium described earlier. In the example below tetrahydropyran-4-carboxylic acid[9] (480 g dissolved in the minimum volume of methanol) was added after 96 hours and again after 168 hours (120 g).

Isolation of Crude Avermectins

The broth was harvested at 288 hours and treated with Hyflo (Johns Manville) (5 kg) and filtered on a plate and frame press. The filtered solids were slurried with acetone (410 litres) for 16 hours and filtered. This procedure was repeated with fresh acetone (410 litres) and the combined acetone extracts were evaporated to 150 litres and the residue extracted with ethyl acetate (3 × 205 litres). The organic layer was separated and evaporated to 10 litres, methanol (10 litres) was added and the resulting solution extracted with hexane (20 litres). The methanol layer was separated and concentrated to 5 litres. The hexane layer was backwashed with 5% aqueous methanol (20 litres) and the methanol layer separated and concentrated to 5 litres. The combined methanol layers were then evaporated to a dark oil (708 g). To the oil in dichloromethane (3.5 litres) was added silica gel (1 kg) and activated carbon (0.5 kg) and the mixture stirred for 2 hours. Then the mixture was filtered and the filter cake washed with dichloromethane (5 litres) and the combined dichloromethane solutions evaporated to a brown oil (494 g). This oil was dissolved in diisopropyl ether (500 ml) and dripped into ice-cooled hexane (18 litres) with vigorous stirring. The mixture was allowed to stand overnight at 4°C and the precipitated solid was separated, dissolved in acetone (1 litre) and evaporated to a foam (65.1 g). The foam was dissolved in dichloromethane (3.9 litres) and stirred for 2 hours with basic alumina (325 g). The mixture was filtered and the filtrate stirred with a further portion of alumina (108 g) for 1 hour and filtered. The alumina filter cakes were combined and stirred with methanol (6 litres) for 30 minutes then filtered. The filtrate was evaporated and the residue taken up in diisopropyl ether (100 ml). This solution was dripped into hexane (1 litre) with vigorous stirring, after 30 minutes the resultant precipitate was filtered off to yield 15 g of pale brown solid.

Preparative Chromatography

The above solid was chromatographed on a Waters Prep 500 instrument using a silica column (50 mm × 50 cm) eluting with a mixture of ethyl acetate and hexane (5 : 4) at 150 ml/minute. Relevant fractions were combined to give 2 enriched mixtures; Fraction A contained 25-(4-tetrahydropyranyl)-avermectins A1 and A2 (3 g), Fraction B contained 25-(4-tetrahydropyranyl)avermectins B1 and B2 (4 g). The individual avermectins were purified as follows and their physical properties are listed in Table 2.

25-(4-Tetrahydropyranyl)avermectin A1 (**1**)

600 mg of Fraction A was chromatographed on a C-18, 8 μm, Dynamax column (41.4 mm × 25 cm) eluting with a mixture of methanol and water (70 : 30) at a flow rate of 45 ml/minute. Relevant fractions were combined to yield the title compound (61 mg).

25-(4-Tetrahydropyranyl)avermectin A2 (**2**)

Relevant fractions from the above chromatography were combined to yield 289 mg of the title compound.

25-(4-Tetrahydropyranyl)avermectin B1 (**3**)

2 g of Fraction B was chromatographed on a C-18, 8 μm, Dynamax column (41.4 mm × 25 cm) eluting with a mixture of methanol and water (85 : 15) at a flow rate of 60 ml/minute. Relevant fraction were combined to yield 1.3 g of the title compound.

Table 2. Physical and spectroscopic data for novel C-25 avermectins.

C-25 substituent	Compound No.	Subclass	MP (°C)	Theoretical MW	$(M+Na)^+$ from FAB-MS	m/z for principle fragments in the EI-MS
4-Tetrahydropyranyl	**1**	A1	175～176	914	937	608, 333, 275, 249, 221, 197, 145, 127, 113, 95, 87
	2	A2	220 (dec)	932	955	351, 333, 275, 267, 249, 239, 221, 197, 145, 127, 113, 95, 87
	3	B1	117～183	900	923	594, 333, 249, 197, 145, 127, 113, 95, 87
	4	B2	173～178	918	941	612, 351, 333, 267, 261, 249, 239, 221, 207, 197, 145, 127, 113, 95, 87
2-Methylcyclopropyl	**5**	A2	147～150	902	925	596, 454, 303, 275, 237, 219, 209, 191, 179, 167, 145, 122, 113, 111, 95, 87
Cyclobutyl	**6**	A1	120～124	884	907	578, 303, 275, 257, 219, 191, 167, 145, 127, 113, 111, 95, 87
	7	A2	135～140	902	925	596, 454, 321, 303, 275, 237, 219, 209, 191, 179, 167, 145, 127, 113, 111, 95, 87
	8	B1	135～138	870	893	303, 261, 257, 219, 191, 167, 145, 127, 113, 111, 95, 87
	9	B2	110～112	888	911	321, 303, 261, 257, 237, 219, 209, 191, 179, 167, 145, 127, 113, 111, 95, 87
Cyclopentyl	**10**	A2	150.5～151	916	939	335, 317, 275, 257, 251, 233, 205, 181, 179, 145, 127, 113, 111, 95, 87
	11	B1	158～162	884	907	578, 468, 317, 257, 251, 233, 205, 181, 179, 145, 127, 113, 111, 95, 87
	12	B2	158～162	902	925	596, 468, 335, 317, 257, 251, 233, 179, 145, 127, 113, 95, 87
Cyclohexyl	**13**	A1	110～115	912	935	606, 331, 275, 257, 247, 218, 195, 145, 127, 113, 95, 87
	14	A2	112～117	930	953	624, 482, 349, 331, 275, 265, 247, 237, 219, 207, 195, 179, 145, 127, 113, 111, 95, 87
	15	B1	116～119	898	921	592, 331, 257, 247, 219, 195, 145, 127, 113, 95, 87
	16	B2	146～148	916	939	610, 482, 349, 331, 275, 265, 257, 179, 145, 127, 113, 95, 87
2-Thienyl	**17**	B1	152～154	898	921	592, 331, 257, 247, 219, 195, 145, 127, 113, 95, 87
3-Thienyl	**18**	A2	167	930	953	349, 331, 275, 265, 257, 247, 237, 219, 195, 145, 127, 113, 95, 87
	19	B1	155～165	898	921	610, 592, 574, 482, 331, 261, 257, 247, 219, 195, 145, 127, 113, 95, 87
	20	B2	175～180	916	939	610, 331, 257, 249, 234, 219, 195, 145, 127, 113, 95, 87
3-Furanyl	**21**	A2	148～153	914	937	333, 315, 275, 257. 249, 231, 221, 203, 179, 145, 127, 113, 95, 87
	22	B1	145～150	882	905	576, 315, 261, 257, 231, 203, 179, 145, 127, 113, 95, 87
3,4-Dihydropyran-2-yl	**23**	A1	130～135	912	935	331, 275, 257, 247, 219, 195, 145, 127, 113, 95, 87

Table 2. (Continued)

C-25 substituent	Compound No.	Subclass	MP (°C)	Theoretical MW	$(M+Na)^+$ from FAB-MS	m/z for principle fragments in the EI-MS
3-Cyclopentenyl	**24**	B1	150~152	882	905	576, 315, 261, 257, 248, 239, 231, 211, 203, 179, 145, 127, 113, 95, 87
3-Cyclohexenyl	**25**	A2	131~135	928	951	624, 486, 347, 329, 275, 263, 245, 235, 217, 205, 193, 179, 145, 127, 113, 111, 95, 87
	26	B1	122~129	896	919	590, 329, 257, 245, 217, 193, 145, 127, 113, 95, 87
1-Methylbutyl	**27**	A1	—	900	923	594, 470, 319, 275, 257, 207, 183, 145, 127, 113, 95, 87
	28	B1	148~150	880	909	580, 337, 319, 261, 257, 253, 225, 207, 183, 145, 127, 113, 111, 95, 87
4-Exomethylene-cyclohexyl	**29**	B1	161~165	910	933	604, 343, 261, 259, 231, 207, 145, 127, 113, 95, 87
1-Methylthioethyl	**30**	A2	134~138	922	945	341, 323, 275, 263, 257, 239, 211, 187, 179, 145, 127, 113, 95, 87
	31	B1	144~147	890	913	584, 232, 261, 257, 239, 211, 187, 145, 127, 113, 95, 87
1-Methyl-3-methoxy propyl	**32**	B1	143~150	902	925	596, 335, 257, 251, 223, 199, 145, 127, 113, 95, 87
1-Methylbut-1-enyl	**33**	B1	140~145	884	907	578, 317, 261, 257, 233, 205, 145, 127, 113, 95, 87
	34	B2	155~160	902	925	596, 578, 335, 317, 262, 257, 233, 205, 145, 127, 95, 87
1-Methylbut-3-ynyl	**35**	B1	95~100	882	905	576, 466, 315, 261, 257, 231, 203, 179, 145, 127, 113, 95, 87
	36	B2	107~110	900	923	594, 466, 333, 315, 261, 257, 249, 231, 221, 203, 179, 145, 127, 113, 95, 87

25-(4-Tetrahydropyranyl)avermectin B2 (**4**)

Relevant fractions from the above chromatography were combined to yield 450 mg of the title compound.

The purities of the above compounds were estimated to be >97%, based on the area under the peak at 243 nm, determined by the previously described HPLC assay.

The same procedure was used to produce a range of avermectins with various groups at C-25 by feeding the relevant carboxylic acid.

Characterization

FAB-MS was performed on a VG Model 7070E mass spectrometer using a sample matrix of triethylene glycol with solid sodium chloride. EI-MS was performed using a VG Model 7070F mass spectrometer. MP's were determined on a Kofler hot-stage apparatus and are uncorrected. The mass spectra of the natural avermectins have been described previously[10] and the novel avermectins exhibited the same characteristic fragmentation patterns. Fragments containing the novel C-25 groups had the appropriate mass difference.

Results

When *S. avermitilis* ATCC 53568 was grown in the absence of supplementary branched-chain carboxylic acids, no avermectins were produced. However when such acids were added to the fermentation, novel

avermectins possessing the relevant acid-derived substituent at the C-25 position were produced. The HPLC assay gave reproducible Rt's with standard deviations of less than 0.2 minute. The order of elution of the four components, namely B2, A2, B1 and A1, remained constant regardless of the nature of the C-25 substituent. Hence the order of elution and characteristic UV chromophores of the avermectins allowed preliminary structural assignments as listed in Table 1. These assignments were then confirmed by scaling-up the shake-flask fermentations and isolating and characterising the avermectins, the analytical data are given in Table 2. The antiparasitic activities of the novel compounds were then assessed as described below.

Antiparasitic Activity

Anthelmintic activity was evaluated against *Caenorhabditis elegans* using an *in vitro* test[11]. All the avermectins in Table 2 killed 100% of the worms at a well concentration of 0.1 μg/ml. Activity against the larval stage of the blowfly *Lucilia cuprina* (Q strain) was demonstrated using a procedure in which first instar larvae were kept in contact with filter paper which had been treated with an acetone solution of the test compound. The treated filter papers were then placed in tubes containing 1 ml of newborn calf serum and the first instars were added. All the avermectins listed in Table 2 killed 100% of the larvae when applied to the filter paper at a level of 1 mg/m^2. Taken together these tests demonstrate the potential of the novel avermectins for broad-spectrum antiparasitic activity.

Discussion

Mutational biosynthesis has been used before to produce analogues of natural products[12] and the production of new antibiotics using this technique has been reviewed[6]. There is some precedent from work on the biosynthesis of fatty acids in *Bacillus subtilis* for incorporation of unnatural cyclic acids as chain initiators[13]. However, in all other previous cases of macrolide biosynthesis the unnatural substrate has taken part in a late or terminal biosynthetic step whereas in our work the substrate is incorporated at an early stage in the biosynthetic sequence leading to the avermectins. Indeed the precursors to the C-25 substituent may be chain initiators, and are therefore incorporated at the very beginning of the polyketide pathway. By analogy to the biosyntheses of erythromycin and tylosine[14,15], chain elongation would then take place by sequential addition of acetate or propionate units to the carboxylic acid chain initiator in parallel with adjustments of the level of oxygenation, followed by macrolactonisation. This being the case, the novel substrate and the biosynthetic intermediates containing the unnatural group must be accepted by most of the enzymes involved in avermectin biosynthesis. It is remarkable then, that the wide range of carboxylic acids listed in Table 1 are all capable of taking part in the biosynthetic pathway to produce avermectins with novel C-25 substituents.

The successful precursor substrates listed in Table 1 were obtained by feeding a large number (>800) of other potential precursors to fermentations of *S. avermitilis*. Relative incorporation was estimated by the area under the new peak at 243 nm using the previously described HPLC assay. Some general conclusions about what kind of carboxylic acids incorporate to produce novel avermectins are listed below.

Eight-carbon acids appear to be the largest that will incorporate. The preference for smaller acids may indicate that certain of the avermectin biosynthetic enzymes recognize the C-25 substituent in a binding region of limited size.

Double bonds and triple bonds are accepted and oxygen or sulfur atoms are tolerated if they are in the form of ethers or contained in aromatic rings. It appears, therefore, that the binding region is small and hydrophobic. Thus polar functional groups such as hydroxyl or amino prevent binding. However an alternative explanation could be that polar acids are not transported efficiently into the cell. The primer transacylase of fatty acid biosynthesis shows a similar specificity in certain organisms[13].

Some insights can be gained into the metabolic capabilities of *S. avermitilis* from the results of feeding carboxylic acid precursors. For example the same novel avermectins are produced when either cyclobutane methanol or cyclobutane carboxylic acid is fed. This illustrates the ability of the microorganism to oxidize primary alcohols to the corresponding carboxylic acids. Similarly, esters or amides may be hydrolysed to their parent acids. Interestingly, amines can also be converted to the corresponding acid, presumably *via* transamination to the aldehyde followed by oxidation.

Carboxylic acids lacking an α-branch can be metabolized by the enzymes of fatty acid β-oxidation. For example if cyclopentane propionic acid is fed, C-25 cyclopentyl avermectins are produced. This probably occurs by dehydrogenation to form cyclopentane acrylic acid, followed by hydroxylation to yield the β-hydroxy acid. The latter is oxidized and cleaved to give cyclopentane carboxylic acid. This process is analogous to catabolism of naturally occurring straight-chain fatty acids[16].

In summary, in this work we have shown that avermectins with novel C-25 substituents can be produced by feeding carboxylic acids or their precursors to a fermentation of a mutant strain of *S. avermitilis* which does not produce avermectins unless so fed. The novel avermectins produced by substrate feeding all possess broad-spectrum antiparasitic activity *in vitro*. In principle, the technique of mutational biosynthesis should be applicable to many other polyketide-derived natural products and in the future may prove to be a rich source of novel analogues.

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