NEW ANTIBIOTICS FROM GENETICALLY ENGINEERED ACTINOMYCETES

I. 2-NORERYTHROMYCINS, ISOLATION AND STRUCTURAL DETERMINATIONS

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Novel macrolide antibiotics have been isolated from a genetically manipulated actinomycete. The major components produced have been isolated and identified as 2-norerythromycins A, B, C and D by mass spectrometry and extensive 1D and 2D NMR experiments.

The potential of genetic engineering to construct plasmid-bearing actinomycetes capable of producing novel antibiotics was demonstrated by HOPWOOD and his group in a collaborative effort with ŌMURA and The Kitasato Institute.[1,2] They inserted genes, coding for the production of actinorhodin, from *Streptomyces coelicolor* into a plasmid and used this to transform mutant strains of *Streptomyces* sp. AM-7161 (the medermycin producer) and of *Streptomyces violaceoruber* Tü 22 (the granaticin producer). From the resulting transformants they were able to produce, isolate and characterize three new antibiotics, mederrhodins A and B and dihydrogranatirhodin.

We have sought to use these techniques to produce novel members of the commercially important class of antibiotics, the macrolides.

A genomic library of DNA from the oleandomycin producer, *Streptomyces antibioticus*, in 30 to 35 kilobase fragments, was inserted into a plasmid, pNJ1, which carried a gene for thiostrepton-resistance. These constructs were then used to transform a mutant of *Saccharopolyspora erythraea* (formerly called *Streptomyces erythraeus*) which was blocked in erythromycin production at some undetermined step prior to lactone synthesis. One out of 288 thiostrepton-resistant clones produced antibiotic activity as detected by agar zone diffusion against *Staphylococcus aureus* (thiostrepton-resistant). The molecular biology of this recombinant will be the subject of a separate article. This clone was grown in submerged fermentation, in the presence of thiostrepton, and the extractable basic antibiotics were isolated and separated by extensive countercurrent chromatography using the Ito Coil Planet Centrifuge.[3]

The macrolides have well resolved ^{1}H NMR spectra and with the simultaneous use of the two-dimensional (2D) techniques ^{1}H-^{1}H correlation spectroscopy (COSY) and ^{1}H-^{13}C chemical shift correlation map (CSCM) it is possible to unambiguously assign every carbon and proton resonance and to give a first order analysis of almost all of the ^{1}H-^{1}H couplings.[4] These analyses, in conjunction with confirmatory IR and mass spectral results, lead to the rapid assignment of structure.

Discussion

The isolation of the macrolide complex from fermentation beers is facilitated by the basic nature

Table 1. Two-phase systems for the separation of macrolides by countercurrent chromatography.

System	Components*	Proportions (by volume)
A	*n*-Heptane - C_6H_6 - 2-PrOH - Me_2CO - H_2O	5 : 10 : 3 : 2 : 5
B	CCl_4 - MeOH - H_2O	1 : 1 : 1
C	$CHCl_3$ - MeOH - H_2O	1 : 1 : 1
D	Hexane - EtOAc - H_2O	3 : 7 : 5

* The aqueous component was a 0.01 M-buffer chosen according to pH.

Buffer	pH range
HOAc - NaOAc	5.0~5.7
Citric acid - Na citrate	5.7~6.5
K_2HPO_4 - KH_2PO_4	6.5~7.5

of these relatively lipophilic molecules. A crude extract containing predominantly macrolides is obtained readily by a simple pH dependent extraction procedure. However, separation of the individual components of a macrolide complex requires high resolution separation techniques. We have developed a series of two-phase solvent systems for countercurrent chromatography using the Ito Coil Planet Centrifuge (Table 1) and effected the required separations with high recovery yields. The partition coefficients, and hence column retention of specific components, could be altered within any one system by adjustment of the pH of the aqueous phase. Acetic acid - sodium acetate, citric acid - sodium citrate, and mono and dibasic potassium hydrogen phosphate buffers were used for pH values in the vicinity of 5, 6 and 7, respectively. The *pKa* of erythromycin in 66% dimethylformamide - water has been reported as 8.6[5] and as such macrolides containing an unmodified desosamine sugar moiety might be expected to be essentially fully protonated within the pH ranges that we have used. Nonetheless, minor changes in pH brought about major differences in the retention of components. For example, erythromycin E eluted from the coil in system D 45 to 57 ml after loading (*i.e.*, close to the solvent front) when the buffer was at pH 7.2, whereas it did not elute until 350 ml after loading with the same system buffered to pH 6.0. Two possible explanations are advanced. Either the *pKa* of the dimethylamino group of desosamine in this complex solvent system is very different from the value determined in 66% dimethylformamide - water or alternatively minute differences in the degree of ionization can effect large changes in the partition coefficients in these multi-component two-phase solvent systems.

Similar TLC mobilities and color reactions with most applicable spray reagents for many macrolide congeners leads to the choice of an ^1H NMR spectrum as the preferred criterion of purity as well as a major tool in structure determination.

From a 750-liter fermentation we have isolated and identified four major macrolide products and five minor congeners. Several other minor macrolide products were also seen but have not been identified.

The four major products were readily characterized by their ^1H NMR spectra as analogs of erythromycins A, B, C and D which lack the methyl group at the 2-position of the macrolide ring. Although not strictly accurate, the names 2-norerythromycin A, B, C and D seem to combine simplicity with descriptiveness for structures **1**, **2**, **3** and **4**.

Detailed parameters from these spectra are listed in Tables 2 and 3. The assignments have been made by consideration of 2D COSY maps and ^1H-^{13}C CSCM experiments which allow for virtually unambiguous assignment of signals.

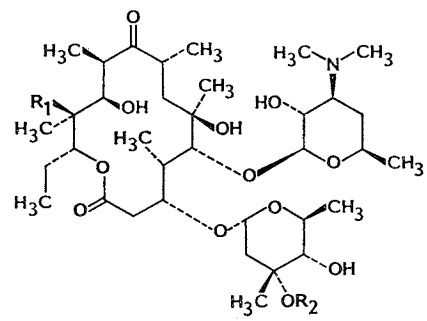
For simplicity the proton spectrum of the erythromycin D analog will be discussed. This spectrum differed from the spectra of the other 2-norerythromycins only significantly by those changes which would be anticipated from *O*-methylation at the 3″-hydroxyl group and/or hydroxylation at C-12 of the macrolide. The ^1H NMR spectrum of 2-norerythromycin D indicated immediately that the structure of this antibiotic differed from the known erythromycins by lack of a *C*-methyl group. The region

from 0.8 to 1.5 ppm downfield from TMS contained one triplet, two singlets and only six doublets arising from aliphatic *C*-methyl groups. The signal at δ 4.10, assigned to 3-H, clearly indicated that this proton was coupled to three other protons and implicated the C(2) methyl or the C(4) methyl as that being absent. Following the coupling pattern either with single frequency decoupling experiments or from a 2D COSY experiment showed that two of these were to a methylene at δ 2.48 and 2.63 which was not further coupled while the third was to a methine at δ 2.21 which in turn was coupled to a methyl at δ 1.13 and a methine at δ 3.50. This sequence established the presumptive structure of 2-norerythromycin D. The structure and those of the congeners were confirmed by ^{13}C NMR spectra. These data are presented in Table 4 and again the assignments, even of close adjacent signals, could be made without ambiguity from the corresponding CSCM experiments. The expected multiplicities are in agreement with the results of DEPT (distortionless enhancement by polarization transfer) experiments.

Fig. 1 shows the difference in chemical shift values for each carbon for 2-norerythromycin A and erythromycin A. As expected, the major differences in chemical shift values for these two compounds occur at C(2) and carbons in the immediate vicinity.

The mass spectral data was also in agreement with these structural assignments. Each of the congeners gave a protonated molecular ion, in the fast atom bombardment (FAB) mode, 14 daltons less than would have been expected from the corresponding erythromycin. In the electron impact (EI) mode the base peak was invariable at *m/z* 158 as is characteristic of desosamine-containing macrolides. In each case the assigned molecular ion was mass-matched to 10^{-4} daltons and the results are in good agreement with that required by the assigned formula.

The *in vitro* antibiotic activity of the 2-norerythromycins is shown in Table 5. These results



2-Norerythromycin A (**1**) R_1 = OH R_2 = CH_3
2-Norerythromycin B (**2**) R_1 = H R_2 = CH_3
2-Norerythromycin C (**3**) R_1 = OH R_2 = H
2-Norerythromycin D (**4**) R_1 = R_2 = H

Table 2. ^1H NMR chemical shift (δ) assignments.

	2-Norerythromycins					2-Norerythromycins			
	A	B	C	D		A	B	C	D
2-H_a	2.52	2.49	2.52	2.48	12-CH_3	1.11	0.87	1.14	0.87
2-H_b	2.59	2.59	2.67	2.63	15-H_3	0.82	0.88	0.84	0.88
3-H	4.05	4.08	4.22	4.10	1′-H	4.31	4.53	4.27	4.20
4-H	2.14	2.26	2.16	2.21	2′-H	3.23	3.25	3.21	3.17
5-H	3.56	3.56	3.53	3.50	3′-H	2.43	2.52	2.45	2.45
7-H_a	1.91	1.99	1.92	1.95	4′-H_a	~1.64	~1.65	1.64	1.64
7-H_b	1.64	1.63	1.71	1.61	4′-H_b	~1.20	~1.20	1.21	1.18
8-H	2.68	2.69	2.67	2.65	5′-H	3.53	3.54	3.53	3.52
10-H	3.12	3.04	3.10	2.96	6′-H_3	1.17	1.21	1.23	1.20
11-H	3.82	3.79	3.80	3.74	$N(CH_3)_2$	2.27	2.35	2.29	2.33
12-H	—	1.67	—	1.62	1″-H	4.82	4.82	5.02	4.97
13-H	5.13	5.45	5.11	5.40	2″-H_a	2.19	2.20	1.97	1.94
14-H_a	1.92	1.71	1.92	1.68	2″-H_b	1.52	1.61	1.83	1.82
14-H_b	1.44	1.48	1.46	1.44	4″-H	3.05	3.01	3.00	2.96
4-CH_3	1.10	1.12	1.11	1.13	5″-H	4.01	4.02	3.83	3.82
6-CH_3	1.43	1.45	1.48	1.46	6″-H_3	1.21	1.25	1.32	1.30
8-CH_3	1.13	1.12	1.18	1.16	3″-CH_3	1.23	1.25	1.27	1.26
10-CH_3	1.14	1.01	1.18	1.00	OCH_3	3.31	3.32	—	—

Table 3. ^{1}H-^{1}H couplings (Hz) of 2-norerythromycins.

J	2-Norerythromycins			
	A	B	C	D
2-H_a, 2-H_b	11.6	11.9	12.0	12.3
2-H_a, 3	2.6	2.7	2.8	2.8
2-H_b, 3	12.3	11.0	11.2	12.3
3, 4	1.6	1.5	~1.0	1.9
4, 4-CH_3	7.0	7.2	7.7	7.1
4, 5	6.8	6.4	7.1	6.6
7-H_a, 7-H_b	15.0	14.6	15.3	14.5
7-H_a, 8	11.3	11.3	11.3	~11.3
7-H_b, 8	1.7	~2.0	~2.5	1.6
8, 8-CH_3	6.9	7.0	—	6.9
10, 11	~1.0	~1.0	~1.0	1.0
10, 10-CH_3	6.9	6.9	6.9	6.9
11, 12	—	10.0	—	9.6
12, 12-CH_3	—	6.8	—	6.9
12, 13	—	1.7	—	~1.0
13, 14-H_a	2.4	4.8	2.1	4.8
13, 14-H_b	10.9	9.4	10.9	9.3
14-H_a, 14-H_b	13.9	13.8	—	—
14-H_a, 15-H_3	7.2	7.4	7.2	7.4
1′, 2′	7.4	7.4	7.2	7.2
2′, 3′	10.0	10.1	10.3	10.2
3′, 4′-H_a	11.1	—	~10.5	11.2
3′, 4′-H_b	3.9	—	~4.0	3.0
4′-H_a, 4′-H_b	—	—	~10.5	13.2
4′-H_a, 5′	—	~2.4	~2.5	3.6
4′-H_b, 5′	—	~11.0	~11.0	11.8
5′, 6′-H_3	6.2	6.2	6.1	6.1
1″, 2″-H_a	~1.0	~1.0	~1.0	~1.0
1″, 2″-H_b	4.7	4.8	3.9	3.5
2″-H_a, 2″-H_b	15.0	15.2	14.5	14.6
4″, 5″	9.7	9.3	9.6	9.5
5″, 6″-H_3	6.4	6.1	6.2	6.2

Table 4. ^{13}C NMR spectral data.

Assignment	2-Norerythromycins				Assignment	2-Norerythromycins			
	A	B	C	D		A	B	C	D
1	170.5	170.9	170.1	170.5	12-CH_3	16.2	9.9	16.2	9.7
2	36.3	36.6	37.3	37.5	15	10.6	10.4	10.7	10.4
3	76.3	76.6	77.2	78.2	1′	102.1	102.1	103.8	103.7
4	36.7	36.7	37.1	37.2	2′	70.9	70.9	70.6	70.6
5	83.5	83.4	84.0	83.9	3′	65.6	65.6	65.4	65.5
6	74.5	74.8	74.5	75.1	4′	28.6	28.6	28.3	28.3
7	38.1	37.7	38.5	38.0	5′	68.7	68.7	69.4	69.3
8	45.5	45.5	45.5	45.4	6′	21.3	21.7	21.3	21.3
9	222.0	221.0	222.3	220.9	$N(CH_3)_2$	40.3	40.3	40.2	40.1
10	37.2	37.8	37.4	38.0	1″	92.2	92.4	94.6	94.8
11	68.8	69.2	68.8	69.3	2″	35.5	35.5	41.3	41.3
12	74.4	40.0	74.7	39.8	3″	73.0	73.0	69.6	69.6
13	77.3	75.0	77.2	75.3	4″	78.1	78.1	76.6	76.3
14	21.0	25.7	21.0	25.6	5″	65.3	65.4	66.5	66.5
4-CH_3	8.5	8.6	8.5	8.5	6″	17.9	17.9	18.0	18.0
6-CH_3	26.5	26.9	26.7	26.9	3″-CH_3	21.7	21.3	25.6	25.6
8-CH_3	18.3	18.4	18.3	18.4	OCH_3	49.3	49.3	—	—
10-CH_3	12.3	9.0	12.1	9.0					

Fig. 1. Differences in ^{13}C NMR values from erythromycin A to 2-norerythromycin A.

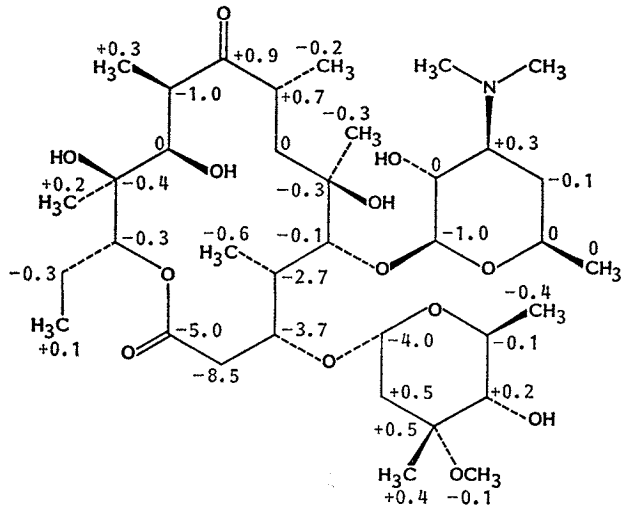


Table 5. *In vitro* activity of 2-norerythromycins (MIC, μg/ml).

Organism	2-Norerythromycins				Erythromycin A
	A	B	C	D	
Staphylococcus aureus ATCC 6538	6.2	6.2	12.5	12.5	0.2
S. aureus CMX 686B	6.2	12.5	25	12.5	0.2
S. aureus A5177	100	>100	>100	>100	1.56
S. aureus 45	6.2	6.2	25	12.5	0.1
S. aureus 45 RAR2	6.2	12.5	25	12.5	0.2
S. aureus CMX 503A	6.2	12.5	25	12.5	0.2
S. aureus CMX 553	6.2	12.5	25	12.5	0.2
S. epidermidis 3519	6.2	12.5	25	12.5	0.2
Micrococcus luteus ATCC 4698	1.56	0.39	1.56	1.56	0.1
Enterococcus faecium ATCC 8043	1.56	3.1	3.1	3.1	0.05
Streptococcus bovis A5169	0.39	0.39	1.56	0.78	0.02
S. agalactiae CMX 508	0.39	0.78	1.56	1.56	0.05
S. pyogenes EES61	0.39	0.78	1.56	1.56	0.02
S. pyogenes 930	>100	>100	>100	>100	>100
S. pyogenes 2548	>25	6.2	25	25	1.56
Escherichia coli Juhl	>100	>100	>100	>100	25
E. coli SS	1.56	3.1	6.2	12.5	0.39
Enterobacter aerogenes ATCC 13048	>100	>100	>100	>100	100
Klebsiella pneumoniae ATCC 8045	>100	>100	>100	>100	50
Providencia stuartii CMX 640	>100	>100	>100	>100	>100
Pseudomonas aeruginosa BHM10	>100	>100	>100	>100	50
P. aeruginosa K799/61	12.5	25	25	50	0.78
P. cepacia 2961	>100	>100	>100	>100	>100
Acinetobacter sp. CMX 669	100	>100	>100	>100	3.1

are perhaps somewhat surprising and disappointing. They indicate that the lack of a methyl group in the C(2) position of the macrolide ring brought about a 20-fold decrease in potency of these compounds with no change in relative antibacterial spectrum. Nonetheless, this example further demonstrates the potential of rDNA techniques to produce novel antibiotics. The 2-norerythromycins provide an unpredictable structure-activity relationship which would have required a gargantuan effort to acquire through synthetic or semisynthetic methods.

Experimental

Fermentation

Frozen mycelia (stored in 30% glycerol at −20°C) were used to inoculate a 2-liter flask containing 500 ml of SCM medium supplemented with 2 μg/ml of thiostrepton. The flask was incubated at 32°C for 96 hours on a rotary shaker at 200 rpm. The resulting culture was used to inoculate 10 liters of the same medium in a LSL Biolafitte fermentor. The fermentation was maintained at pH 7.0 with 5 M NaOH and 5 M acetic acid and was aerated at 20 liters per minute and stirred at 500 rpm. SCM media consisted of soluble starch 15 g/liter, Soytone 20 g/liter, $CaCl_2$ 0.1 g/liter, yeast extract 1.5 g/liter, soy oil 50 ml/liter and MOPS 10.5 g/liter. The crude media is adjusted to pH 7.0 with 1 M KOH before autoclaving.

Harvest

After 168 hours the fermentation was harvested. The pH was raised to 9.4 with 1 M KOH and the medium was centrifuged at 7,000×*g* for 15 minutes. Residual oil was decanted and discarded. The aqueous supernate was decanted and the mycelial pellet was resuspended in an equal volume of acetone. The acetone suspension was mixed thoroughly and centrifuged at 7,000×*g* for 10 minutes. The supernate was decanted and concentrated under reduced pressure to a semisolid residue. This was digested in water, adjusted to pH 9.4 and added to the aqueous beer supernate. The combined aqueous mixture was extracted with EtOAc (3×0.4 volumes) and the combined EtOAc extracts were concentrated to *ca.* 150 ml. This was extracted with 2% aqueous citric acid (3×60 ml). The combined extracts were adjusted to pH 9.4 with 1 M NaOH solution and reextracted with EtOAc (3×100 ml). The combined EtOAc extracts were dried (Na_2SO_4) and concentrated under reduced pressure to a solid residue.

Scale-up

A 750-liter fermentation was similarly performed with the contents of 3×10 liters fermentors at 96 hours age as the inoculum. This, after a similar harvest to that described above for the 10 liters fermentation, gave ~4 g of EtOAc extractable basics.

Isolation

Scheme 1 describes the isolation of individual macrolide components from the extract obtained from a 750-liter fermentation.

Spectral Measurements

IR spectra were measured on $CDCl_3$ solutions with a Perkin-Elmer 683 dual beam dispersive instrument in the fourier transformation (FT) mode. NMR spectra were measured on samples in $CDCl_3$ with a General Electric GN-500 spectrometer operating at 500.10 MHz and 125.74 MHz for ^1H and ^{13}C, respectively. Phase sensitive double quantum filtered COSY spectra were collected using the method of STATES *et al.*[6] in 2K×330 point hypercomplex data matrix zero filled to 2K×2K. Absolute value heteronuclear correlation spectra with broad-band proton decoupling in F1 and F2 were collected in an 8K×100-point complex data matrix zero filled to 8K×256 data points. Homonuclear *J*-resolved spectra were collected in a 4K×80-point complex data matrix zero filled to 8K×256 data points. Mass spectra were measured on a Kratos MS-50 spectrometer in either the EI or FAB mode.

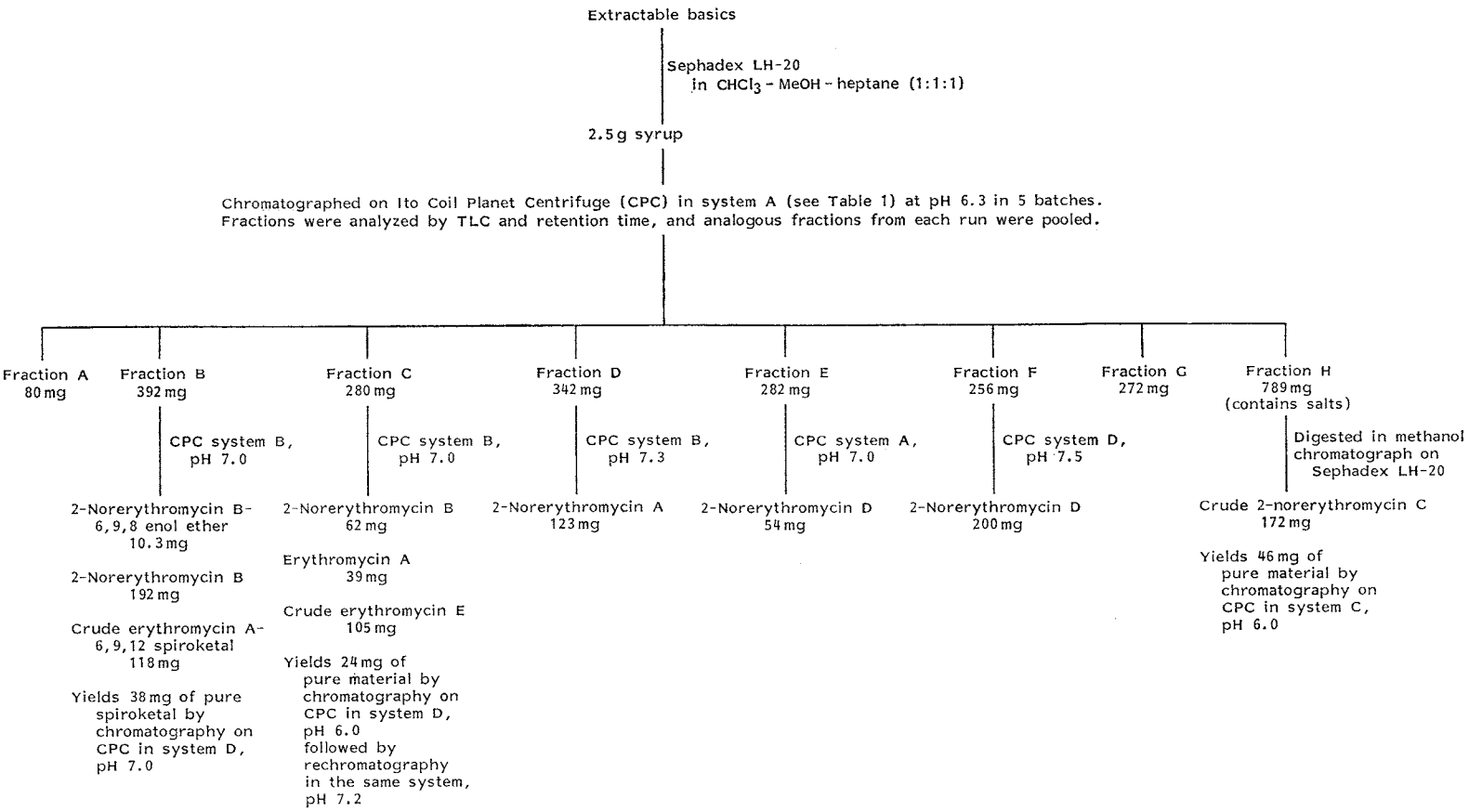
Isolated Compounds

2-Norerythromycin A: 123 mg obtained as a white foam had ^1H and ^{13}C NMR spectra as described in Tables 2, 3 and 4. MS, FAB positive ion *m/z* 720, EI *m/z* 719.4431 $C_{36}H_{65}NO_{13}$ requires *m/z* 719.4456, base peak at *m/z* 158; $[\alpha]_D^{26}$ −64° (*c* 0.87, MeOH); IR ν_{max} cm^{-1} 3600, 3520, 1718, 1690.

2-Norerythromycin B: Total 254 mg obtained as a white foam had ^1H and ^{13}C NMR spectra as described in Tables 2, 3 and 4. MS, FAB positive ion *m/z* 704, EI *m/z* 703.4535 $C_{36}H_{65}NO_{12}$ requires *m/z* 703.4503, base peak at *m/z* 158; $[\alpha]_D^{26}$ −100° (*c* 0.29, MeOH); IR ν_{max} cm^{-1} 3600, 3520, 1718, 1690.

2-Norerythromycin C: 46 mg of analytically pure material obtained as a clear glass had ^1H and ^{13}C NMR spectra as described in Tables 2, 3 and 4. MS, FAB positive ion *m/z* 706, EI *m/z* 705.4330

Scheme 1. Isolation of 2-norerythromycins.



$C_{35}H_{63}NO_{13}$ requires *m/z* 705.4299, base peak at *m/z* 158; $[\alpha]_D^{26}$ −58° (*c* 0.32, MeOH); IR ν_{max} cm^{-1} 3600, 3520, 1720, 1682.

2-Norerythromycin D: 254 mg obtained as a white foam had ^1H and ^{13}C NMR spectra as described in Tables 2, 3 and 4. MS, FAB positive ion *m/z* 690, EI strong peak at *m/z* 671 $[M-H_2O]^+$, mass-matched at *m/z* 671.4246 $C_{35}H_{61}NO_{11}$ requires 671.4245, base peak at *m/z* 158; $[\alpha]_D^{26}$ −72° (*c* 0.43, MeOH); IR ν_{max} cm^{-1} 3600, 3520, 3500, 1720, 1680.

In Vitro-antimicrobial Assay

Minimal inhibitory concentrations (MICs) were determined using the standard agar dilution procedure[7] on brain heart infusion agar.

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