VOL. 54 NO. 1, JAN. 2001 THE JOURNAL OF ANTIBIOTICS pp. 1 — 9

Production, Isolation and Structure Determination of Novel Fluoroindolocarbazoles from *Saccharothrix aerocolonigenes* ATCC 39243

KIN SING LAM*, DANIEL R. SCHROEDER, JACQUELINE M. VEITCH, KIMBERLY L. COLSON, JAMES A. MATSON, WILLIAM C. ROSE, TERRENCE W. DOYLE and SALVATORE FORENZA

Bristol-Myers Squibb Company, Pharmaceutical Research Institute, P.O. Box 5100, Wallingford, CT 06492, U.S.A.

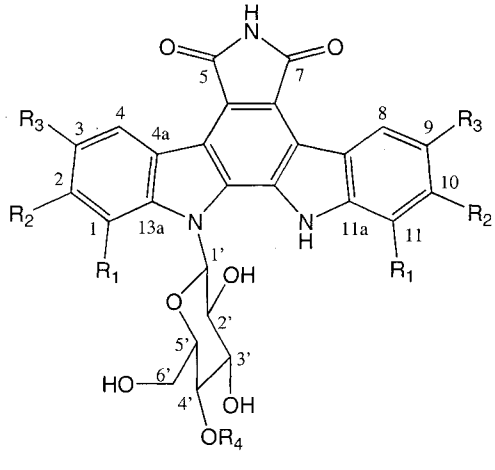
(Received for publication June 26, 2000)

Saccharothrix aerocolonigenes ATCC 39243 produces an indolocarbazole antitumor agent rebeccamycin under submerged fermentation conditions. Adding DL-6-fluorotryptophan to culture of *S. aerocolonigenes* ATCC 39243 induces the formation of two novel indolocarbazoles, fluoroindolocarbazoles A and B. Feeding DL-5-fluorotryptophan to culture of *S. aerocolonigenes* ATCC 39243 induces the production of a novel indolocarbazole, fluoroindolocarbazole C. These fluoroindolocarbazoles have been isolated from culture broth and purified to homogeneity by vacuum liquid chromatography and column chromatography. All three fluoroindolocarbazoles are more potent than rebeccamycin against P388 leukemia by ip route in murine model.

Rebeccamycin is an indolocarbazole antitumor antibiotic isolated from culture of *S. aerocolonigenes* ATCC 39243[1]. Rebeccamycin has a broad spectrum of *in vivo* activity in tumor-bearing murine models[1]. It consists of a chlorinated indolocarbazole chromophore to which attached a 4′-*O*-methylglucose *via* an *N*-glycosidic linkage (Fig. 1). The novel chemotype shows good potential to yield candidates for development toward clinical trials for cancer treatment in humans. A water-soluble analog of rebeccamycin, BMY-27557, was prepared by chemical modification of the imide nitrogen of rebeccamycin[2,3]. BMY-27557 is more potent than rebeccamycin against P388 leukemia in murine model[3]. BMY-27557 is currently undergoing Phase II clinical trials for treatment of heptatobiliary and gall bladder cancers.

KANEKO *et al.*[2] devised a chemical protocol for the preparation of haloindolocarbazoles. An alternative to this chemical approach is directed biosynthesis. Biosynthetic studies demonstrated that rebeccamycin is derived from two molecules of tryptophan, one of glucose and one of methionine[4]. In a previous paper[5], we reported the production and isolation of a novel bromoindolocarbazole

Fig. 1. The structures of rebeccamycin and fluoroindolocarbazoles.



	R_1	R_2	R_3	R_4
Rebeccamycin	Cl	H	H	CH_3
Fluoroindolocarbazole A	H	F	H	CH_3
Fluoroindolocarbazole B	H	F	H	H
Fluoroindolocarbazole C	H	H	F	H

* Corresponding: Kin.Lam@bms.com

from culture of *S. aerocolonigenes* ATCC 39243 supplemented with 0.05% potassium bromide in a defined medium. However, addition of potassium fluoride to the defined medium did not lead to the production of the corresponding fluorinated analog. The haloperoxidase that catalyzes the halogenation reaction may not have an oxidation-potential sufficient to oxidize fluoride ions[6] before they could be incorporated into rebeccamycin. There are numerous publications reporting the preparation of fluorinated metabolites by feeding the microorganisms with fluorinated precursors[7~14]. In particular, fluorinated analogs of pyrrolnitrin[7], actinomycin[11], asperlicin[13] have been prepared by feeding the microorganisms with fluorotryptophans. Since the indolocarbazole moiety of rebeccamycin is derived from tryptophan, it is likely that the addition of fluorotryptophans to cultures of *S. aerocolonigenes* ATCC 39243 induces the formation of new fluoroindolocarbazoles.

The fluorine atom has the strongest electronegativity among all the elements and the C–F bond energy is greater than that of the C–H or C–Cl bond[15]. The fluorinated derivatives may therefore possess different biological activities as compared to the parent compound. In this paper, we describe the production, isolation and structure determination of three fluoroindolocarbazoles from cultures of *S. aerocolonigenes* ATCC 39243 supplemented with DL-5-fluorotryptophan or DL-6-fluorotryptophan.

Materials and Methods

General Procedures

The UV spectra were recorded on a Hewlett-Packard 8452A diode array spectrophotometer. IR spectra were recorded on Perkin-Elmer 1800 FTIR spectrometer. FAB-MS spectra were obtained on a Kratos MS 25 mass spectrometer. ^{1}H-NMR (360 MHz) and ^{13}C-NMR (90 MHz) spectra were obtained on a Bruker model AM-3000 spectrometer. Additional NMR spectra were acquired on a Bruker AM-500 operating at 500.13 mHz at 298K. The spectra were obtained in "100%" DMSO-d_6 and referenced to this solvent. Long range heteronuclear coupling information was obtained using COLOC experiments utilizing mixing times of 0.06 and 0.08 s. The coupling constants were determined by direct measurement from the one-dimensional spectra. The number of attached protons to each carbon was determined by a DEPT-135 experiment.

Microorganism

The rebeccamycin-producing microorganism was *Saccharothrix aerocolonigenes* ATCC 39243. Frozen vegetative preparations were maintained in 10% glycerol-5% sucrose solution stored at −80°C for use as working stocks.

Media and Fermentation Conditions

To prepare an inoculum for the production phase, 4 ml of the frozen vegetative stock was transferred to a 500-ml flask containing 100 ml of vegetative medium consisting of glucose 3%, Pharmamedia 1%, Nutrisoy 1% and $CaCO_3$ 0.3%. This vegetative culture was incubated at 28°C on a rotary shaker at 250 rpm. After 48 hours, 3-ml aliquots were transferred to 500-ml flasks containing 100 ml of medium DF-36. Medium DF-36 consists of starch 1%, L-threonine 0.25%, $MgSO_4 \cdot 7H_2O$ 0.1%, KH_2PO_4 0.2% and $CaCO_3$ 0.2%. The production cultures were incubated at 28°C and 250 rpm for 6 days. For the production of fluoroindolocarbazoles, DL-5-fluorotryptophan or DL-6-fluorotryptophan was added to the production cultures at a final concentration of 1 mg/ml at 48 hours.

Extraction and Analytical Methods

The production of rebeccamycin and fluoroindolocarbazoles were monitored by HPLC using a C-18 reversed-phase column (μBondapak, 3.9×300 mm, Waters Associates) and UV absorption at 313 nm. The solvent system was 0.1 M $NH_4OAc/CH_3OH/CH_3CN$ (4 : 3 : 3) with a flow rate of 1.5 ml/minute. Fermentation extracts for HPLC analysis were prepared by centrifuging 3 ml of fermentation broth at 1,500×g for 15 minutes. The supernatant was discarded and the mycelial mat was extracted with 1 ml of acetone. The extract was dried under N_2 and stored at −20°C until use. The extract was dissolved in 1 ml of DMSO and 50 μl of the solution was used for HPLC analysis. The HPLC system (Hewlett-Packard) was equipped with a diode array detector which provided UV-Vis spectra (200~600 nm) of the metabolites contained in the crude extracts.

Isolation of Fluoroindolocarbazoles A and B

Two liters culture broth of *S. aerocolonigenes* ATCC 39243 supplemented with DL-6-fluorotryptophan was filtered on diatomaceous earth (Dicalite). The resulting mycelial mat was suspended in 2 liters of THF, stirred for 45 minutes and filtered again. The Dicalite was further rinsed with THF until it no longer fluoresced yellow under UV light (365 nm). The combined filtrates were concentrated under reduced pressure to yield 3.39 g of crude extract. The crude extract was triturated with five 50-ml volumes of THF, which were combined and

concentrated to yield 0.74 g of THF soluble residue. This THF soluble material was absorbed onto 2 g Silica Gel H (Merck, 10~40 μm) and applied to a 30 ml sintered glass funnel (medium porosity) containing an additional 11 g Silica Gel H. A hexane-ethyl acetate step gradient was carried out using 100-ml volumes of eluent. The two major rebeccamycin analogs were separated cleanly in this manner. The less polar yellow analog (141 mg) eluted with hexane - ethyl acetate (1 : 1) and the more polar analog (105 mg) with hexane - ethyl acetate (1 : 3).

The less polar analog (141 mg) was dissolved in 2 ml of methanol and applied to a column containing 110 g Sephadex LH-20 pre-swollen in methanol (2.5 cm i.d., bed height 80 cm, bed volume 400 ml). The column was eluted with methanol at a flow rate of 3.5 ml/minute. The main yellow zone eluting at the end of the fourth bed volumes was collected. Slow addition of hexane to this solution precipitated 70 mg of a bright yellow solid designated as fluoroindolocarbazole A.

The more polar analog (105 mg) was dissolved in 2 ml of THF and applied to a column containing 160 g Sephadex LH-20 (2.5 cm i.d., bed height 90 cm, bed volume 430 ml) pre-swollen in THF. The column was eluted with THF at a flow rate of 4 ml/minute. The main yellow band was collected that eluted at 1.25 bed volumes. Slow addition of hexane to this solution caused precipitation of a bright yellow solid (77 mg) designated as fluoroindolocarbazole B.

Isolation of Fluoroindolocarbazole C

Five liters culture broth of *S. aerocolonigenes* ATCC 39243 supplemented with DL-5-fluorotryptophan was filtered on Dicalite. The resulting mycelial mat was suspended in 2 liters of THF, stirred for 1 hour and filtered again. The Dicalite was further rinsed with 1 liter of THF. The combined filtrates were concentrated under reduced pressure to yield 4.5 g of crude extract. The extract was adsorbed onto 6.5 g Lichroprep Si 60 silica gel (EM Science, 15~25 μm) and applied to a 60 ml VLC funnel containing an additional 24.5 g silica gel. A hexane-ethyl acetate step gradient was carried out (200 ml volumes) to 100% ethyl acetate, followed by a 200 ml volume THF wash. The THF wash contained the yellow band, which was evaporated to dryness to yield 156 mg residue. This residue was dissolved in 4 ml of THF and applied to a column containing 160 g Sephadex LH-20 pre-swollen in THF (2.5 cm i.d., bed height 90 cm, bed volume 430 ml). The column was eluted with THF at a flow rate of 3.75 ml/minute. A major yellow band was collected that eluted at 1.2 bed volumes. This fraction was evaporated to dryness to yield 62 mg residue. Final purification was accomplished by semi-preparative HPLC using a Whatman Partisil 10 ODS-3 column (10 mm×50 cm) and an isocratic solvent system consisting of 0.1 M NH_4OAc/THF (3 : 2). At a flow rate of 4 ml/minute, the major rebeccamycin analog was eluted at 39 minutes. The fractions containing this analog were pooled, and upon drying yielded 23 mg of bright yellow solid designated as fluoroindolocarbazole C.

Antitumor Assay

The general procedures used to assess antitumor activity using the P388 leukemia model have been described[16,17]. Specifically, in the tests described herein, female (BALB/c×DBA/2) F_1 hybrid mice were implanted intraperitonelly (ip) with 10^6 P388 cells and compounds were administered ip on day 1 post-implant only. There were four to six mice in each treatment group and 10 mice in the control groups. Each compound was administered at several dose levels, usually at 50% decrements. Efficacy was evaluated by determining the relative median life spans of treated (T) and control mice and expressing the ratios (after multiplying by 100) in terms of %T/C values. A T/C value of ≥125% was considered active.

Results

Production of Fluoroindolocarbazoles by Directed Biosynthesis

During the course of our studies on the regulation and biosynthesis of rebeccamycin by *S. aerocolonigenes* ATCC 39243[4,18], a defined medium DF-36 was developed for the production of rebeccamycin. When DL-6-fluorotryptophan was added at day 2 of the fermentation to yield a final concentration of 1 mg/ml, two new major metabolites (fluoroindolocarbazoles A and B) with Rts of 15.7 and 22.4 minutes respectively, were detected in the mycelial extract by HPLC analysis. The UV spectra of these metabolites (Table 1 and 2) were very similar to that of rebeccamycin indicating that these metabolites have similar chromophores as rebeccamycin. Rebeccamycin (Rt of 18.1 minutes) was not detected in the above fermentation by HPLC analysis. When DL-5-fluorotryptophan was added at day 2 of the fermentation yielding a final concentration of 1 mg/ml, one major metabolite (fluoroindolocarbazole C) with Rt of 9.9 minutes was detected in the mycelial extract by HPLC analysis. This new metabolite also has a UV spectrum (Table 3) similar to rebeccamycin and is another new analog.

Table 1. Physico-chemical properties of fluoroindolocarbazole A.

Appearance	Bright yellow amorphous solid
MW	537
Molecular formula	$C_{27}H_{21}F_2N_3O_7$
FAB-MS (m/z)	538 $(M + H)^+$, 361 (M – 176)
UV λ^{MeOH} nm (ε)	228 (780), 256 (454), 280 (387), 316 (1050), 398 (103)
IR (cm^{-1})	3324, 2938, 1747, 1703, 1623, 1580, 1491, 1471, 1452, 1412, 1384, 1330, 1233, 1172, 1140, 1116, 1087, 1054, 963, 918, 828, 764, 649, 635, 618, 498
^{1}H-NMR (360 MHz) Obsd chemical shifts (ppm)	11.77 (s, 1H), 11.18 (s, 1H), 9.12 (dd, 1H), 9.05 (dd, 1H), 7.88 (dd, 1H), 7.41 (dd, 1H), 7.23 (m, 2H), 6.25 (d, 1H), 6.24 (s, 1H), 5.36 (dd, 1H), 5.31 (d, 1H), 5.04 (d, 1H), 3.97 (m, 2H), 3.60-3.95 (m, 4H)
^{13}C-NMR[a] (90 MHz) Obsd chemical shifts (ppm)	170.8, 170.7, 161.8 (d), 161.7 (d), 143.1 (d), 141.5 (d), 130.1, 128.7, 126.0 (d), 125.9 (d), 120.9, 119.4, 118.2, 118.1, 117.8, 116.6, 108.8 (d), 108.6 (d), 98.8 (d), 98.3 (d), 84.4, 77.2, 77.2, 76.2, 73.2, 59.9, 58.5
TLC[b](Rf)	0.72

[a] Proton decoupled spectrum: d = doublet indicate fluorine coupling
[b] Silica gel plate (Silica gel 60, Merck): ethyl acetate

Physico-chemical Properties and Structure Determination of Fluoroindolocarbazole A

The physico-chemical properties of fluoroindolocarbazole A are summarized in Table 1. Fluoroindolocarbazole A was soluble in DMSO, DMF, THF and acetone but only sparingly soluble in ethyl acetate and methanol. In general, it has considerably improved solubility properties as compared to the parent compound rebeccamycin, which only showed good solubility in THF. Observed in the FAB mass spectrum for fluoroindolocarbazole A was an $(M+H)^+$ ion of 538 without the characteristic isotopic cluster seen with rebeccamycin indicating the absence of the two chlorine atoms. The MW of fluoroindolocarbazole A corresponds to the replacement of two chlorine atoms on the aglycone of rebeccamycin with two fluorine atoms. The mass spectrum also showed a prominent fragment ion at *m/z* 361 corresponding to the aglycone with the loss of 4′-*O*-methylglucose. The same facile cleavage was observed in the mass spectrum of rebeccamycin. The chemical shifts for the ^{1}H-NMR and proton-decoupled ^{13}C-NMR of fluoroindolocarbazole A are shown in Table 1. The NMR data for the aglycone portion of fluoroindolocarbazole A is nearly identical with the data presented below for the aglycone of fluoroindolocarbazole B which was the focus of a more rigorous treatment involving complete assignment of resonances. NMR data for the sugar portion, including coupling constants, could be compared directly with the 4′-*O*-methylglucose portion of rebeccamycin. Based on the spectral data, it was readily determined that fluoroindolocarbazole A has the structure 2,10-difluoro-1,11-dideschloro-rebeccamycin as shown in Fig. 1.

Physico-chemical Properties of Fluoroindolocarbazole B

The physico-chemical properties of fluoroindolocarbazole B are summarized in Table 2. Fluoroindolocarbazole B has a similar solubility profile as fluoroindolocarbazole A except for an increased solubility in methanol and other lower alcohols. The FAB mass spectrum for fluoroindolocarbazole B indicated 524 as the $(M+H)^+$ ion, 14 Daltons less than was observed for

Table 2. Physico-chemical properties of fluoroindolocarbazole B.

Appearance	Bright yellow amorphous solid
MW	523
Molecular formula	$C_{26}H_{19}F_2N_3O_7$
FAB-MS (m/z)	524 $(M + H)^+$, 361 (M – 162)
UV λ^{MeOH} nm (ε)	226 (526), 256 (313), 280 (259), 316 (640), 398 (60)
IR (cm^{-1})	3324, 2927, 1745, 1701, 1623, 1580, 1491, 1471, 1452, 1412, 1384, 1330, 1233, 1172, 1116, 1075, 1048, 1016, 963, 916, 829, 764, 745, 646, 635, 617, 498, 490
TLC[a] (Rf)	0.4

[a] Silica gel plate (Silica gel 60, Merck): ethyl acetate

fluoroindolocarbazole A, and suggesting the absence of a methyl group. The same prominent fragment ion at *m/z* 361 was observed in fluoroindolocarbazoles A and B indicating the aglycone portions had the same MW. Comparisons of UV and IR spectra for fluoroindolocarbazoles A and B also indicated that their respective aglycones had identical substitution patterns. The 14 Daltons difference was therefore thought to reside in the glucose appendages, and NMR work confirmed this supposition.

The chemical shift assignments for fluoroindolocarbazole B were determined using COSY, HETCOR, COLOC, DEPT and selective irradiation experiments. NMR chemical shift data for proton and carbon resonance's are summarized in Table 3 along with C–F and C–H coupling constants. The carbons bearing fluorine (C-2, C-10) were easily distinguished by the large 240 Hz coupling constants. The fluorine-carbon *ortho* and *meta* coupling constants also greatly assisted in the assignments of the aromatic resonances. Long range proton-carbon couplings, determined by COLOC experiments, defined the positions of several quaternary carbons. However, the nearly identical chemical shifts of some carbon resonances required a greater resolution for assignment than was possible using the two-dimensional COLOC experiment, thus selective proton decoupled carbon experiments were acquired. Even with that effort some resonances (imide carbonyls at 171.0 and 171.1, and adjacent carbons at 121.0 and 119.4 ppm) could not be distinguished and therefore were not positively assigned. In d_6-DMSO solution, the imide and indole exchangeable NH resonances were very sharp and proved to be invaluable in assigning several quaternary carbons and distinguishing the two halves of the indolocarbazole aglycone.

Assignment of resonances for the sugar portion was made using the COSY experiment aided by HETCOR and COLOC experiments. The only problem encountered for the sugar was the assignments of carbon resonances at 67.6 and 78.7 ppm. The corresponding methine protons both resonate at 3.95 ppm (4′-H and 5′-H) and the neighboring proton at 3.83 ppm (6′-H) showed long range couplings to both of these carbons, confusing the issue. Hence, a comparison to a model compound β-D-glucopyranose[19] was necessary to assign the carbon chemical shifts. Based on the spectral data, it was determined that fluoroindolocarbazole B has the structure 2,10-difluoro-1,11-dideschloro-4′-*O*-desmethylrebeccamycin as shown in Fig. 1.

Physico-chemical Properties and Structure Determination of Fluoroindolocarbazole C

The physico-chemical properties of fluoroindolocarbazole C are summarized in Table 4. By a similar line of reasoning used for deducing the structures of fluoroindolocarbazoles A and B, the structure of fluoroindolocarbazole C was determined to be 3,9-difluoro-1,11-dideschloro-4′-*O*-desmethylrebeccamycin as shown in Fig. 1. The full assignment of NMR resonances was not undertaken for this

Table 3. Complete NMR assignments for fluoroindolocarbazole B.

Carbon	Chemical shift (ppm)	J C-F (Hz)	Attached Proton	Multiplicity	J H (H or F) (Hz)	^{1}JC-H (Hz)	COLOC data
1	99.0	27.9	7.88	dd	10.6, 2.2	166.1	
2	161.9	240.2					7.41, 7.88
3	109.0	24.4	7.23	m		163.9	7.88
4	126.2	16.5	9.06	dd	8.5, 5.8	166.6	
4a	117.8						7.88
4b	118.2						
4c	121.0						11.17
5	171.0						11.17
6-NH			11.17	s			
7	171.1						11.17
7a	119.4						11.17
7b	116.6						11.17
7c	118.3						7.41, 11.17
8	126.0	16.2	9.12	dd	8.8, 6.0	166.6	
9	108.8	24.3	7.23	m		163.9	7.41
10	161.9	240.2					7.41, 7.88
11	98.5	26.3	7.41	dd	9.23, 2.3	166.4	
11a	141.7	13.2					9.12, 11.77
12-NH			11.77	s			
12a	130.2						11.77
12b	128.8						6.25
13 (N)							7.88, 9.06
13a	143.3	13.1					7.88, 9.06
1'	84.8		6.25	d	8.6	157.6	
2'	73.2		3.47	ddd	12.8, 5.4, 8.6	143.6	
3'	76.5		3.58	ddd	12.8	140.4	
4'	67.6		3.95	m		141.1	3.83
5'	78.7		3.95	m		144.1	3.83
6'	58.4		3.83	bd	11.6	143.2	
			4.09	dd	11.6, 3.2		
2'-OH			4.96	d	5.4		
3'-OH			5.14	bs			
4'-OH			5.41	bs			
6'-OH			6.11	t	3.2		

dd: doublet of doublets, m: multiplet, bs: broad singlet, bd: broad doublet, t: triplet, s: singlet, ddd: doublet of doublet of doublets

analog.

Antitumor Activity

The effects of fluoroindolocarbazoles A and B on mice bearing P388 leukemia are shown in Table 5. Both compounds produced active results with maximum T/C values of 206% and 178%, respectively. Fluoroindolocarbazole B was tested on two occasions and the maximum effects achieved (170% and 178%) were consistent. The dose levels that caused the maximum effects of these compounds were approximately 50 mg/kg for fluoroindolocarbazole A and 100 mg/kg for fluoroindolocarbazole B. Notably, for both compounds, the lowest dose evaluated of each, 0.8 mg/kg, was also associated with active results, T/C values of 140~156%. In comparison, concomitant testing with rebeccamycin led to maximum T/C values of 172~175%, comparable

Table 4. Physico-chemical properties of fluoroindolocarbazole C.

Appearance	Bright yellow amorphous solid
MW	523
Molecular formula	$C_{26}H_{19}F_2N_3O_7$
FAB-MS (m/z)	524 $(M + H)^+$, 361 (M – 162)
UV λ^{MeOH} nm (ε)	230 (348), 259, 277, 288, 322 (457), 405
IR (cm^{-1})	3340, 2915, 1752, 1708, 1628, 1591, 1484, 1462, 1392, 1331, 1292, 1247, 1191, 1112, 1081, 1052, 947, 904, 795, 762, 752, 511
^1H NMR (360 MHz) Obsd chemical shifts (ppm)	11.76 (s, 1H), 11.23 (s, 1H), 8.85 (dd, 1H), 8.77 (dd, 1H), 8.01 (dd, 1H), 7.68 (dd, 1H), 7.46 (m, 2H), 6.29 (d, 1H), 6.12 (t, 1H), 5.45 (d, 1H), 5.17 (d, 1H), 4.95 (d, 1H), 4.07 (d, 1H), 3.95 (m, 2H), 3.81 (d, 1H), 3.59 (m, 2H)
^{13}C NMR[a] (90 MHz) Obsd chemical shifts (ppm)	170.9, 170.8, 157.0 (d), 157.0 (d), 138.6, 137.2, 130.7, 129.2, 121.7 (d), 121.4 (d), 121.3, 119.6, 117.9, 116.6, 115.0 (d), 114.6 (d), 113.3 (d), 113.2 (d), 109.1 (d), 109.1 (d), 84.8, 78.7, 76.5, 73.2, 67.6, 58.3
TLC[b] (Rf)	0.1

[a] Proton decoupled spectrum: d = doublet indicates fluorine coupling
[b] Silica gel plate (Silica gel 60, Merck): ethyl acetate

to fluoroindolocarbazole B in both experiments, and slightly less than the maximum effect obtained with fluoroindolocarbazole A. These maximum effects of rebeccamycin were obtained at the highest doses of the compound evaluated, 240~300 mg/kg, 2.4 to 6-fold greater than the optimal doses of the analogs. With regard to comparative minimal effective doses of rebeccamycin and the two derivatives, these cannot be accurately be discerned because the doses of rebeccamycin evaluated were not titrated to inactive levels. Historically, however, rebeccamycin has been found to be inactive on occasion at 32 mg/kg[1], or minimally effective at 20 mg/kg (W. C. ROSE; unpublished data). Such data would suggest at least a 20~40 fold difference in minimal effective potency between rebeccamycin and the two analogs.

In a pilot study in which only a cursory evaluation of fluoroindolocarbazole C was performed (using only four mice per treatment group), the derivative achieved a maximum T/C value of 175% at 30 mg/kg, and was active at a dose as low as 10 mg/kg (Table 6). In this experiment, rebeccamycin achieved a maximum T/C value of 155% at 200~300 mg/kg. These data also suggest that a 6.7~10 fold and 2~3 fold difference in optimal doses and minimal effective potency, respectively, between rebeccamycin and fluoroindolocarbazole C.

Discussion

In this publication we report the production of three novel fluoroindolocarbazoles in cultures of *S. aerocolonigenes* ATCC 39243 by directed biosynthesis. We have previously established that tryptophan is one of the biosynthetic precursors for rebeccamycin[4]. A defined medium that shows a distinct trophophase and idiophase has also been developed[4]. The distinct phasing was considered promising for efficient utilization of added precursors or precursor analogs, a postulation that was shown to be correct. Adding DL-6-fluorotryptophan to a culture of *S. aerocolonigenes* ATCC 39243 at the start of idiophase (~2 days) induced the formation of two novel indolocarbazoles, fluoroindolocarbazoles A

Table 5. Effects of fluoroindolocarbazoles A and B on P388 leukemia.

		Derivative		Rebeccamycin	
Compound	Expt No.	Dose (mg/kg/inj)	(%T/C)	Dose (mg/kg/inj)	(%T/C)
Fluoroindolocarbazole B	1	200	150	240	175
		100	170	120	165
		50	155	60	165
		25	140	30	145
		12.5	145		
		6.25	140		
Fluoroindolocarbazole B	2	102.4	178	300	172
		51.2	167	200	167
		25.6	161	100	161
		12.8	167	50	144
		6.4	167		
		3.2	156		
		1.6	156		
		0.8	144		
Fluoroindolocarbazole A	2	102.4	161	300	172
		51.2	206	200	167
		25.6	156	100	161
		12.8	161	50	144
		6.4	178		
		3.2	183		
		1.6	156		
		0.8	156		

All treatments were administered once, ip, on day 1 post-leukemia (10^6 cells, ip) implant. There were 6 mice per treatment group and 10 mice in each saline-treated control group.

and B. Feeding a culture of *S. aerocolonigenes* ATCC 39243 DL-5-fluorotryptophan at the start of idiophase induced the formation of a novel indolocarbazole, fluoroindolocarbazole C. Rebeccamycin was not detected in the above fermentations. The production of new indolocarbazoles in the broth extract can be easily identified by HPLC equipped with a diode array detector.

Three fluoroindolocarbazoles were isolated from the culture broth and the structures determined. The structures of fluoroindolocarbazoles were determined by spectroscopic methods and FAB-MS and are shown in Fig. 1. The purification scheme for the fluoroindolocarbazoles as presented in the Materials and Methods section is simple, suitable for scale up and has good recovery yield.

The fluoroindolocarbazoles reported in this study are more potent than rebeccamycin in the *in vivo* antitumor assay. The replacement of chlorine atoms by fluorine atoms and the positions of the attachment of halogens on the

Table 6. Effect of fluoroindolocarbazole C on P388 leukemia.

Compound	Dose (mg/kg/inj)	Effect (%T/C)
Fluoroindolocarbazole C[a]	90	165
	30	175
	10	145
Rebeccamycin[b]	300	155
	200	155
	100	145
	50	145

[a] Four mice were used in each treatment group.
[b] Six mice were used in each treatment group.

aglycone may explain the increase in activity and potency of the new indolocarbazoles. These fluoroindolocarbazoles have good potential for development as anticancer agents in humans.

References

1) BUSH, J. A.; B. H. LONG, J. J. CATINO, W. T. BRADNER & K. TOMITA: Production and biological activity of rebeccamycin, a novel antitumor agent. J. Antibiotics 40: 668~678, 1987
2) KANEKO, T.; H. S. WONG & J. UTZIG: Rebeccamycin derivative containing pharmaceutical composition. U.S. Patent 4,808,613, Feb. 28, 1989
3) KANEKO, T.; H. WONG, J. UTZIG, J. SCHURIG & T. W. DOYLE: Water soluble derivatives of rebeccamycin. J. Antibiotics 43: 125~127, 1990
4) LAM, K. S.; S. FORENZA, D. R. SCHROEDER, T. W. DOYLE & C. J. PEARCE: Biosynthesis of rebeccamycin, a novel antitumor agent. *In* Novel Microbial Products for Medicine and Agriculture. Vol. 1. *Ed.*, A. L. DEMAIN *et al.*, pp. 63~66, Elsevier, 1989
5) LAM, K. S.; D. R. SCHROEDER, J. M. VEITCH, J. A. MATSON & S. FORENZA: Isolation of a bromo analog of rebeccamycin from *Saccharothrix aerocolonigenes*. J. Antibiotics 44: 934~939, 1991
6) NEIDLEMAN, S. L. & J. GEIGERT: Biohalogenation: Principle, Basic Roles and Applications. P. 100, Ellis Horwood Limited, a division of John Wiley & Sons, 1986
7) HAMILL, R. L.; R. P. ELANDER, J. A. MABE & M. GORMAN: Metabolism of tryptophan by *Pseudomonas aureofaciens*. III. Production of substituted pyrrolnitrins from tryptophan analogues. Appl. Microbiol. 19: 721~725, 1970
8) ISONO, K.; P. F. CRAIN, T. J. ODIORNE, J. A. MCCLOSKEY & R. J. SUHADOLNIK: Biosynthesis of the 5-fluoropolyoxins, aberrant nucleoside antibiotics. J. Am. Chem. Soc. 95: 5788~5789, 1973
9) TOSCANO, L.; G. FLIORIELLO, R. SPAGNOLI, L. CAPPELLETTI & G. ZANUSO: New fluorinated erythromycins obtained by mutasynthesis. J. Antibiotics 36: 1439~1450, 1983
10) KAWASHIMA, A.; H. SETO, M. KATO, K. UCHIDA & N. OTAKE: Preparation of fluorinated antibiotics followed by ^{19}F NMR spectroscopy. I. Fluorinated vulgamycins. J. Antibiotics 38: 1499~1505, 1985
11) KAWASHIMA, A.; H. SETO, M. KATO, A. YASUDA, K. UCHIDA & N. OTAKE: Preparation of fluorinated antibiotics followed by ^{19}F NMR spectroscopy. II. Fluorinated actinomycins. J. Antibiotics 38: 1625~1628, 1985
12) KAWASHIMA, A.; H. SETO, T. ISHIYAMA, M. KATO, K. UCHIDA & N. OTAKE: Fluorinated blasticidin S. Agric. Biol. Chem. 41: 1183~1184, 1987
13) HOUCK, D. R.; J. ONDEYKA, D. L. ZINK, E. INAMINE, M. A. GOETZ & O. D. HENSENS: On the biosynthesis of asperlicin and the directed biosynthesis of analogs in *Aspergillus alliaceus*. J. Antibiotics 38: 882~891, 1988
14) CANNELL, R. J. P.; M. J. DAWSON, R. S. HALE, R. M. HALL, D. NOBLE, S. LYNN & N. L. TAYLOR: The squalestatins, novel inhibitors of squalene synthase produced by a species of *Phoma*. IV. Preparation of fluorinated squalestatins by directed biosynthesis. J. Antibiotics 46: 1381~1389, 1993
15) CHAMBERS, R. D.: Fluorine in Organic Chemistry. P.5, John Wiley and Sons, New York, 1973
16) GERAN, R. I.; N. H. GREENBERG, M. M. MACDONALD, A. M. SCHUMACHER & B. J. ABBOT: Protocols for screening chemical agents and natural products against tumors and other biological systems (3rd edition). Cancer Chemother. Rep. Part 3, 3: 1~103, 1972
17) BRADNER, W. T.: Transplanted animal tumors. *In* Cancer and Chemotherapy. Vol. 1. *Eds.*, S. T. CROOKE & A. W. PRESTAYO, pp. 221~227, Academic Press, New York, 1980
18) LAM, K. S.; J. MATTEI & S. FORENZA: Carbon catabolite regulation of rebeccamycin production in *Saccharothrix aerocolonigenes*. J. Ind. Microbiol. 4: 105~108, 1989
19) VOELTER, W.; V. BILIK & E. BREITMAIER: Konfigurations- und mutarotationseinflusse auf die ^{13}C-NMR spektren einiger disaccharide. Collection Czech. Chem. Commun. 38: 2054~2071, 1973