EPOCARBAZOLINS A AND B, NOVEL 5-LIPOXYGENASE INHIBITORS

TAXONOMY, FERMENTATION, ISOLATION, STRUCTURES AND BIOLOGICAL ACTIVITIES

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New 5-lipoxygenase inhibitors, designated epocarbazolins A and B, were isolated from the culture broth of *Streptomyces anulatus* T688-8. These compounds showed potent rat 5-lipoxygenase inhibitory activity with weak antibacterial activity. Structural studies revealed that epocarbazolins are new carbazole antibiotics having a novel substitution pattern and an epoxide in the side chain.

It is widely known that arachidonic acid-metabolites products, particularly the arachidonate 5-lipoxygenase (5-LPO) metabolites, are observed as chemical mediators in certain inflammatory and allergic states. Moreover, arachidonic acid and its oxidation products were found in psoriatic skin at increased concentrations.[1] Accordingly, 5-LPO inhibitors which block the production of such chemical mediators are expected to be useful for the therapeutic treatment of allergies and inflammations such as asthma, psoriasis and hypersensitivity.

In the course of screening for 5-LPO inhibitors using RBL-1 enzyme, an actinomycete strain identified as *Streptomyces anulatus* T688-8 was found to produce novel 5-LPO inhibitors, designated epocarbazolins A and B.

In this paper, we describe the taxonomy of the producing organism, fermentation, isolation, structure and biological activities of epocarbazolins A and B.

Taxonomy

Strain T688-8 isolated from a soil sample collected in Andhra Pradesh State, India, grew well on both chemically defined media and natural organic media. The substrate mycelium was well-branched and non-fragmentary. The aerial mycelium was monopodially branched and formed chains of spores with more than 30 spores per chain. The spore chains were long, straight or occasionally flexuous (Section: *Rectiflexibiles*). The spores were oblong ($0.5 \sim 1.2 \times 1.0 \sim 2.0\,\mu m$) in shape with smooth surface (Fig. 1). Spore mass color was grayish yellow (Yellow-color series). Sclerotia, sporangia and flagellate spores were not observed. The utilization of carbon compound was examined

Fig. 1. Scanning electron micrograph of spore chains of strain T688-8 grown on ISP-2 medium.

Bar represents $5\,\mu m$.

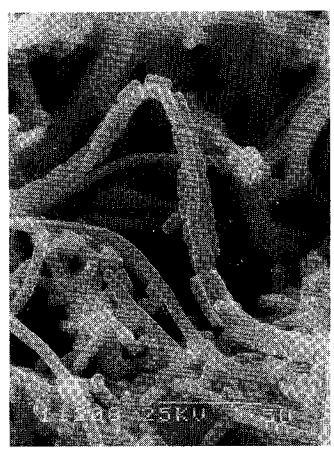


Table 1. Cultural characteristics of strain T688-8.

Medium	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
Sucrose-nitrate agar (CZAPEK-DOX agar)	Good	Good; yellowish gray (93)	Light orange yellow (70)	None
Tryptone-yeast extract broth (ISP No. 1)	Moderate, floccose, not turbid	None	Colorless	Dark brown (59)
Yeast extract-malt extract agar (ISP No. 2)	Good	Good; grayish yellow (90)	Deep yellowish brown (75)	Deep yellowish brown (75)
Oatmeal agar (ISP No. 3)	Poor	Poor; grayish yellow (90)	Colorless	None
Inorganic salts-starch agar (ISP No. 4)	Moderate	Poor; grayish yellow (90)	Light yellowish brown (76)	None
Glycerol-asparagine agar (ISP No. 5)	Moderate	Moderate; white to pale yellow (89)	Light yellow (86)	None
Peptone-yeast extract-iron agar (ISP No. 6)	Moderate	None	Colorless	Dark brown (59)
Tyrosine agar (ISP No. 7)	Moderate	Moderate; yellowish gray (90)	Moderate yellowish brown (77)	None
Glucose-asparagine agar	Moderate	Scant; white	Brilliant yellow (83)	None

Observation after incubation at 28°C for 3 weeks.
Color and number in parenthesis follow ISCC-NBS designation.

Table 2. Physiological characteristics of strain T688-8.

Hydrolysis of:		D-Galactose	+
Gelatin	+	D-Fructose	+
Starch	+	D-Mannose	+
Casein	+	L-Sorbose	−
Production of:		Sucrose	−
Nitrate reductase	+	Lactose	+(w)
Tyrosinase	+	Cellobiose	+
Tolerance to:		Melibiose	−
Lysozyme, 0.01% (w/v)	−	Trehalose	+
NaCl, 0~6% (w/v)	+	Raffinose	−
7%	−	D-Melezitose	−
Temperature:		Dextran	+
Growth range	14~43°C	Soluble starch	+
No growth	12°C and 45°C	Cellulose	−
Utilization of:		Adonitol	−
Glycerol	+	Dulcitol	−
D-Arabinose	−	Inositol	+
L-Arabinose	+	D-Mannitol	+
D-Xylose	+	D-Sorbitol	−
D-Ribose	+	Xylitol	−
L-Rhamnose	+	Salicin	+(w)
D-Glucose	+		

−: Negative, +(w): weakly positive, +: positive.

by the method of PRIDHAM and GOTTLIEB.[2] Cultural and physiological characteristics of strain T688-8 are shown in Tables 1 and 2.

The whole cell hydrolysate contained L-diaminopimelic acid, ribose, mannose and glucose. Phospholipids contained phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol. Therefore, strain T688-8 belongs to cell wall Type I and phospholipid Type P-II.

Based on the above-mentioned characteristics, strain T688-8 was considered to belong to the genus *Streptomyces* Waksman and Henrici, 1943. Among known *Streptomyces* species listed in "Approved List of Bacterial Names",[3~5] strain T688-8 most closely resembled *Streptomyces anulatus* (Beijerinck 1912) Waksman 1957. The taxonomic characteristics of strain T688-8 were compared with those of *S. anulatus*.[6] As shown in Table 3, no significant difference is observed between the two cultures except for melanoid pigment formation and utilization of inositol. Therefore, we concluded that strain T688-8 belonged to *S. anulatus*, and designated it as *S. anulatus* T688-8.

Table 3. Taxonomical comparison of strain T688-8 with *Streptomyces anulatus*.

	Strain T688-8	*S. anulatus* ISP 5361
Spore chain morphology	RF*	RF* or RA**
Spore surface	Smooth	Smooth
Color of Aerial mycelium	Yellow	Yellow or white
Diffusible pigment	None	None
Melanoid pigment formation		
ISP-1	Positive	Negative
ISP-6	Positive	Negative
ISP-7	Negative	Negative
Carbon utilization		
D-Glucose	Positive	Positive
L-Arabinose	Positive	Positive
D-Xylose	Positive	Positive
D-Fructose	Positive	Positive
D-Mannitol	Positive	Positive
Inositol	Positive	Negative or weak positive
L-Rhamnose	Positive	Positive
Raffinose	Negative	Negative or weak positive
Sucrose	Negative	Negative or weak positive

* *Rectiflexibiles*.
** *Retinaculiaperti*.

Fermentation

A loopful spores from a well sporulated slant culture of *S. anulatus* T688-8 were inoculated in 500-ml Erlenmeyer flasks containing 100 ml each of seed medium consisting of soluble starch 2%, glucose 0.5%, NZ-case (Humko Sheffield Chemical) 0.3%, yeast extract (Nihon Seiyaku) 0.2%, fish meal D30X (Banyu Nutrient) 0.5% and $CaCO_3$ 0.3%, the pH being adjusted to 7.0 before sterilization. Shake cultivation was carried out at 28°C for 4 days on a rotary shaker (200 rpm). The seed culture (5 ml) was transferred into 500-ml Erlenmeyer flasks containing 100 ml of production medium consisting of lactose 1%, dextrin 3%, linseed meal 2%, $CaSO_4$ 0.6% and $CaCO_3$ 0.5%, the pH being adjusted to 7.0 before autoclaving. The fermentation was carried out for 112 hours at 28°C and 200 rpm on a rotary shaker.

Isolation

The fermented broth (18 liters) was separated into the mycelial cake and the supernate by filtration. The mycelial cake was extracted twice with methanol (each 4.5 liters) and the methanolic extract was evaporated to give an aqueous solution. The solution was extracted three times with ethyl acetate (each 600 ml) and the extracts were concentrated to dryness *in vacuo* to yield a crude solid (7.0 g). This solid was applied onto a silica gel column (BW-820MH, Fuji Division Chemical Co., Ltd., 200 ml) which was washed with a mixture of dichloromethane-methanol (50 : 1, 3 liters) and then eluted with a mixture of dichloromethane-methanol (4 : 1, 1 liter). Active fractions were examined by inhibitory activity against 5-LPO and/or HPLC analysis. The active eluate fractions were evaporated to dryness and the residue was charged on a reversed-phase C18 column (YMC-ODS, AM type, YMC Co., Ltd., 200 ml) and developed with a mixture of acetonitrile-water (1 : 1, v/v). Active fractions (20 ml each) containing epocarbazolin A (Nos. 21~30) or B (Nos. 37~45) were pooled and combined each. Evaporation of the solvent yielded 44.5 mg of epocarbazolin A and 52.5 mg of epocarbazolin B, respectively. These compounds were photo-labile and all the operations were performed in the dark.

Table 4. Physico-chemical properties of epocarbazolins A and B.

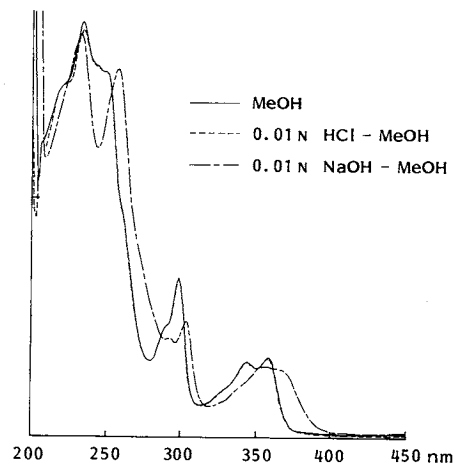
	Epocarbazolin A	Epocarbazolin B
Appearance	Pale yellow powder	Pale yellow powder
MP (°C, dec)	>100[a]	>100[a]
$[\alpha]_D^{26}$ (*c* 0.5, MeOH)	+75°	+78°
FAB-MS (*m/z*)	369 (M^+), 352	383 (M^+), 366
Molecular formula	$C_{22}H_{27}NO_4$	$C_{23}H_{29}NO_4$
HRFAB-MS Calcd:	369.1940	383.2097
Found:	369.1913	383.2209
TLC[b] (Rf value)	0.49	0.49
HPLC[c] (Rt)	3.76 minutes	5.31 minutes
UV λ_{max}^{MeOH} nm (ε)	234 (38,600), 250 (sh, 33,900), 289 (sh, 10,200), 299 (14,800), 345 (7,100), 359 (7,500)	233 (37,900), 250 (sh, 33,900), 289 (sh, 10,200), 300 (14,900), 344 (7,000), 358 (7,300)
IR ν_{max}^{KBr} cm^{-1}	3460, 3430, 2960, 2930, 2870, 1620, 1590, 1525, 1500, 1450, 1390, 1370,1270, 1170, 1120, 1075, 980	3460, 3420, 2960, 2930, 2870, 1620, 1585, 1525, 1500, 1450, 1380, 1270, 1170, 1120, 1075, 985

[a] Could not be determined accurately because of their photo-unstability.
[b] Silica gel plate 60 F_{254} (Merck); CH_2Cl_2-MeOH (9:1).
[c] YMC-Pack A-301-3 (ODS 3 μm, i.d. 4.6×100 mm); CH_3CN-H_2O (55:45); 1.2 ml/minutes.

Physico-chemical Properties

Physico-chemical properties of epocarbazolins A and B are summarized in Table 4. They are soluble in methanol, ethyl acetate and acetone, slightly soluble in chloroform, but practically insoluble in hexane and water. They are unstable to light and alkaline pHs. Color reactions are as follows: Positive response to iodine and sulfuric acid but negative to ninhydrin reagent. Epocarbazolins A and B showed the same UV spectra exhibiting absorption maxima at 234, 250, 289, 299, 345 and 359 nm in methanol, which are closely related to those of carbazomycin A.[7,8] The UV and IR spectra of epocarbazolin B are shown in Figs. 2 and 3, respectively. The ^1H and ^{13}C NMR data of epocarbazolins A and B are shown in Tables 5 and 6, respectively. The ^{13}C data of epocarbazolin B displayed 23 signals composed of CH_3–C×4, –CH_2–×4, >CH–×2, –CH=×3, >C=×9 and a quaternary carbon. In ^1H NMR spectrum of epocarbazolin B, four D_2O exchangeable protons were observed. The molecular formulae of epocarbazolins A and B wer established as $C_{22}H_{27}NO_4$ and $C_{23}H_{29}NO_4$, respectively, on the basis of HRFAB-MS and NMR data.

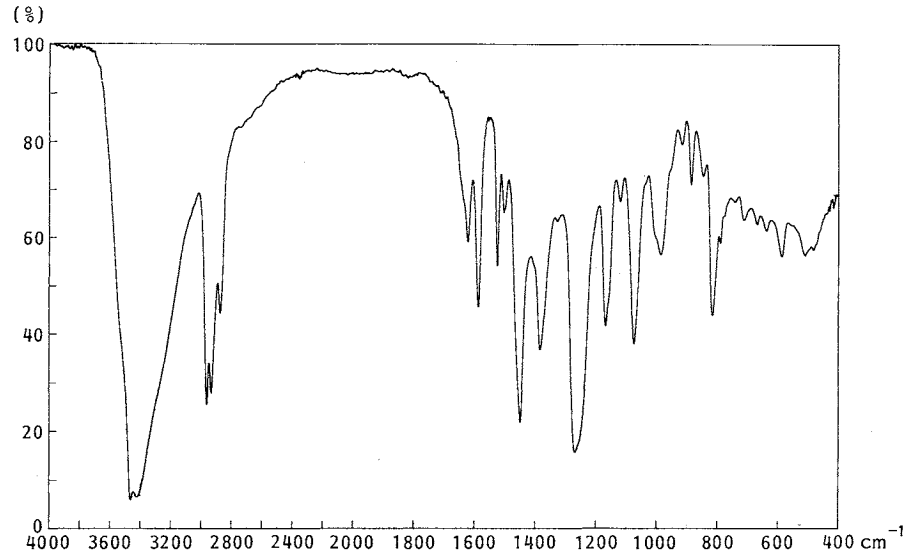
Fig. 2. UV spectrum of epocarbazolin B.



Structure Elucidation

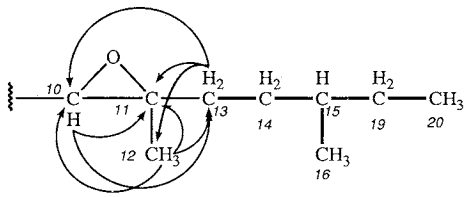
The characteristic UV spectra of epocarbazolins were closely related to that of carbazomycin A, indicating that they share a carbazole nucleus as the chromophore. The ^{13}C NMR spectrum of epocarbazolin B revealed the presence of twelve sp^2 hybridized carbons which are assignable to the

Fig. 3. IR spectrum of epocarbazolin B (KBr disk).



carbazole nucleus (C-1～C-12). In the ^{1}H NMR spectrum of epocarbazolin B, three aromatic protons were observed as AB spin pattern doublet (δ 6.87, 6.71, J=7.7 Hz) and singlet (δ 7.61), suggesting that epocarbazolin B contained the five-substituted carbazole ring.

Fig. 4. Partial structure of epocarbazolin B as revealed by ^{1}H-^{13}C long range COSY.



The ^{1}H-^{1}H COSY and ^{1}H-^{13}C long range COSY experiments of epocarbazolin B disclosed the remaining substituent groups consisting of one hydroxymethyl, one methyl, two hydroxy groups and the alkyl side chain as shown in Fig. 4. The structure of this side chain was determined as follows. The ^{1}H-^{1}H COSY spectra revealed the presence of an 3-methyl-pentyl chain moiety (C-13～C-20). The ^{1}H-^{13}C long range couplings were observed between the end of this alkyl chain 13-H and C-10, C-11, C-12; 12-H and C-10, C-11, C-13; 10-H and C-11, C-13. The presence of an epoxide ring was substantiated by the chemical shifts of C-10 and C-11 (δ_C 64.7 and 63.8, respectively) and the C-H coupling constant of C-10 and 10-H (J=175 Hz).

The position of each substituents on the carbazole nucleus was clarified by NMR spectral analyses of *N*,*O*-tetramethylepocarbazolin B which was prepared by permethylation of epocarbazolin B with MeI in the presence of NaH in DMF.[9] In benzene-d_6, this compound existed as a mixture of unseparable conformers at a ratio of about 3:2, but, their proton and carbon signals were clearly assigned with the aid of the 2D NMR techniques. In the NOESY spectrum, crosspeaks were observed between 3-OCH_3 and 4-H, 4-H and 18-H, 18-H and 6-H, 7-H and 8-OCH_3 and 10-H and 17-CH_3, establishing the position of five substituents as shown in Fig. 5. All the proton and carbon signals were unambiguously assigned by the ^{1}H-^{13}C COSY and ^{1}H-^{13}C long range COSY experiments (Tables 5 and 6). ^{1}H-^{13}C long range correlations (Fig. 5), which also supported the proposed structure.

Epocarbazolin A showed very similar properties to epocarbazolin B, but it differed in the lack of

Fig. 5. ^{1}H-^{13}C long range COSY and NOESY of tetramethylepocarbazolin B.

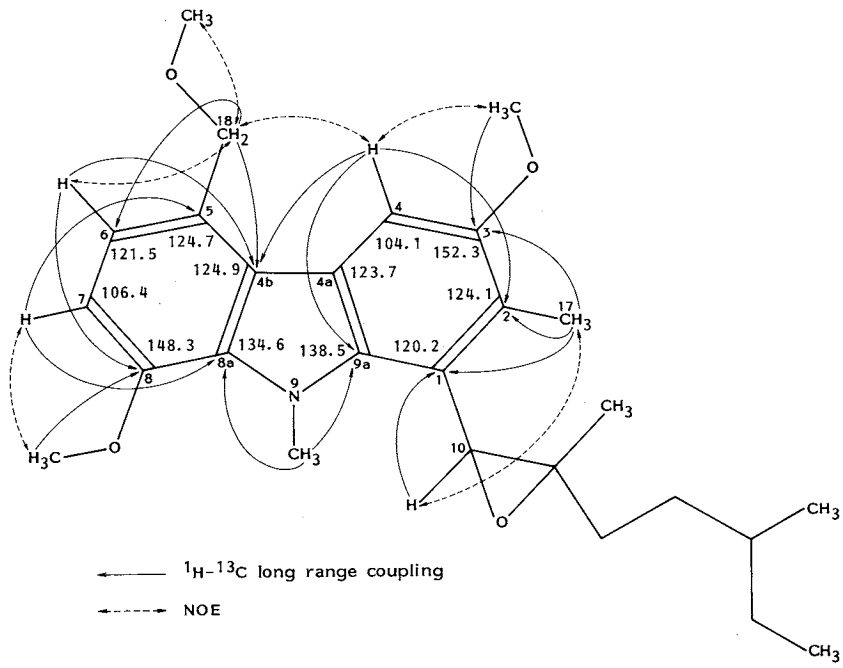


Table 5. ^{1}H chemical shifts of epocarbazolins A and B and tetramethylepocarbazolin B*.

Carbon No.	Epocarbazolin		Tetramethylepocarbazolin B	
	A	B	Major proton	Minor proton
4	7.60 (1H, s)	7.60 (1H, s)	8.03 (1H, s)	7.96 (1H, s)
6	6.87 (1H, d, J=7.7 Hz)	6.87 (1H, d, J=7.7 Hz)	7.09 (1H, d, J=7.7 Hz)	7.14 (1H, d, J=7.7 Hz)
7	6.71 (1H, d, J=7.7 Hz)	6.71 (1H, d, J=7.7 Hz)	6.65 (1H, d, J=7.7 Hz)	6.67 (1H, d, J=7.7 Hz)
10	4.20 (1H, s)	4.21 (1H, s)	4.04 (1H, s)	4.03 (1H, s)
12	1.00 (3H, s)	1.00 (3H, s)	0.99 (3H, s)	0.88 (3H, s)
13	1.80 (2H, m)	1.86 (2H, m)	1.68 (2H, m)	1.77 (2H, m), 1.56
14	1.52 (2H, m), 1.40	1.40 (2H, m), 1.62	1.52 (2H, m), 1.40	1.52 (2H, m), 1.40
15	1.61 (1H, m)	1.40 (1H, m)	1.32 (1H, m)	1.32 (1H, m)
16	0.93 (3H, d, J=6.4 Hz)	0.91 (3H, d, J=6.4 Hz)	0.93 (3H, d, J=7.3 Hz)	0.93 (3H, d, J=7.3 Hz)
17	2.30 (3H, s)	2.30 (3H, s)	2.54 (3H, s)	3.01 (3H, s)
18	4.95 (2H, d, J=5.6 Hz)	4.94 (2H, d, J=5.1 Hz)	4.95 (2H, d, J=11.1 Hz), 5.01	4.97 (2H, s)
19	0.93 (3H, d, J=6.4 Hz)	1.20 (2H, m), 1.37	1.38 (2H, m), 1.16	1.38 (2H, m), 1.16
20	—	0.89 (3H, d, J=6.4 Hz)	0.90 (3H, m)	0.90 (3H, m)
3-OH	8.59 (1H, br s)	8.60 (1H, br s)	—	—
8-OH	7.89 (1H, br s)	7.90 (1H, br s)	—	—
9NH	9.24 (1H, br s)	9.25 (1H, br s)	—	—
18-OH	3.86 (1H, t, J=5.6 Hz)	3.86 (1H, t, J=5.1 Hz)	—	—
3-OCH_3			3.82 (3H, s)	3.76 (3H, s)
8-OCH_3			3.46 (3H, s)	3.52 (3H, s)
9NCH_3			4.42 (3H, s)	4.04 (3H, s)
18-OCH_3			3.32 (3H, s)	3.33 (3H, s)

* Solvent: Epocarbazolins A and B in $(CD_3)_2CO$, tetramethylepocarbazolin B in C_6D_6.

Table 6. ^{13}C chemical shifts of epocarbazolins A and B and tetramethylepocarbazolin B*.

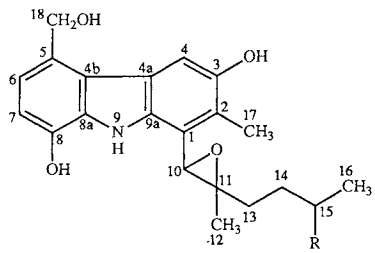
Carbon No.	Epocarbazolin		Tetramethyl-epocarbazolin B		Carbon No.	Epocarbazolin		Tetramethyl-epocarbazolin B	
	A	B	Major carbon	Minor carbon		A	B	Major carbon	Minor carbon
1	118.6 (s)	118.6 (s)	120.2 (s)	120.7 (s)	12	17.5 (q)	17.5 (q)	17.4 (q)	18.1 (q)
2	122.1 (s)	122.1 (s)	124.1 (s)	128.7 (s)	13	36.9 (t)	36.5 (t)	35.6 (t)	34.7 (t)
3	150.1 (s)	150.0 (s)	152.3 (s)	153.5 (s)	14	35.4 (t)	33.0 (t)	31.7 (t)	31.4 (t)
4	108.6 (d)	108.6 (d)	104.1 (d)	104.3 (d)	15	29.5 (d)	35.9 (d)	34.9 (d)	34.9 (d)
4a	123.4 (s)	123.4 (s)	123.7 (s)	122.5 (s)	16	23.3 (q)	20.0 (q)	11.5 (q)	11.5 (q)
4b	123.1 (s)	123.0 (s)	124.9 (s)	125.3 (s)	17	12.9 (q)	12.9 (q)	13.6 (q)	14.5 (q)
5	129.0 (s)	129.0 (s)	124.7 (s)	125.1 (s)	18	64.4 (t)	64.4 (t)	74.0 (t)	73.9 (t)
6	119.6 (d)	119.6 (d)	121.5 (d)	121.7 (d)	19	23.4 (q)	30.4 (t)	29.5 (t)	29.5 (t)
7	110.6 (d)	110.6 (d)	106.6 (d)	106.3 (d)	20	—	121.1 (q)	19.3 (q)	19.3 (q)
8	143.4 (s)	143.4 (s)	148.3 (s)	147.9 (s)	3-OCH_3			55.5 (s)	55.6 (s)
8a	131.1 (s)	131.1 (s)	134.6 (s)	133.5 (s)	8-OCH_3			55.4 (s)	55.2 (s)
9a	134.3 (s)	134.3 (s)	138.5 (s)	137.5 (s)	9NCH_3			36.6 (s)	36.5 (s)
10	64.7 (d)	64.7 (d)	61.7 (d)	61.9 (d)	18-OCH_3			56.9 (s)	57.1 (s)
11	63.8 (s)	63.9 (s)	63.3 (s)	62.5 (s)					

* Solvent: Epocarbazolins A and B in $(CD_3)_2CO$, tetramethylepocarbazolin B in C_6D_6.

Table 7. Antimicrobial activities of epocarbazolins A and B.

Test organism	Epocarbazolin A	Epocarbazolin B
Staphylococcus aureus 6538P	50	12.5
S. aureus Smith	50	12.5
S. aureus Sa-247	50	12.5
Enterococcus faecalis A9808	50	25
Micrococcus luteus ATCC 9341	25	3.1
Bacillus subtilis ATCC 6633	50	12.5
Escherichia coli Juhl	>100	>100
E. coli 255	>100	>100
Klebsiella pneumoniae ATCC 10031	100	>100
Proteus mirabilis IFO 3849	>100	>100
P. vulgaris Pv-44	>100	>100
Serratia marcescens Sm-237	>100	>100
Pseudomonas aeruginosa A9843A	>100	>100

Fig. 6. Structures of epocarbazolins A and B.



Epocarbazolin A R=$-CH_3$
Epocarbazolin B R=$-CH_2CH_3$

Table 8. 5-LPO inhibitory activity of epocarbazolins A and B and carbazomycin B.

Compound	IC_{50} (μM)
Epocarbazolin A	2.4
Epocarbazolin B	2.6
Tetramethylepocarbazolin B	>22.8
Carbazomycin B	1.5

methylene signals in the alkyl side chain based on the ^1H and ^{13}C NMR spectral analyses. Epocarbazolin A had a 2-methyl-butyl group in its side chain instead of the 3-methyl-pentyl group in epocarbazolin B. These differences also supported by the molecular formulae of epocarbazolins A and B. Thus the structures of epocarbazolins A and B have been determined as shown in Fig. 6.

Antimicrobial Activity

The antimicrobial spectra of epocarbazolins A and B were determined *in vitro* by the standard agar dilution method employing nutrient agar medium (Eiken, pH 7.0). MICs were expressed in μg/ml after overnight incubation at 32°C. The antimicrobial activities of epocarbazolins A and B are summarized in Table 7. Both compounds show moderate activity against Gram-positive bacteria but are inactive against the Gram-negative bacteria tested.

5-LPO Inhibitory Activity

The inhibitory activity against 5-LPO was evaluated by the method of D. J. HOOK *et al.*,[10] which comparatively determined the amounts of 5-hydroxy-eicosatetraenoic acid (5-HETE) produced in the enzyme solution containing arachidonic acid, ATP, calcium chloride and glutathione in the presence and absence of inhibitor.

The 5-LPO inhibitory activities of epocarbazolins A and B and tetramethylepocarbazolin B are comparable to that of the known 5-LPO inhibitor carbazomycin B as shown in Table 8.

Discussion

The structure studies have showed that epocarbazolins A and B are new members of the carbazole group of antibiotics. Among the carbazole antibiotics, epocarbazolins A and B are the first carbazole derivatives having the epoxide ring in their molecule, and their 5,8-substituted carbazole nucleus is also very unique.

Epocarbazolins A and B show potent 5-LPO inhibitory activity. Since tetramethylepocarbazolin B has no inhibitory activity against 5-LPO, the presence of a free phenolic hydroxy group seems to be essential for 5-LPO inhibitory activity. Recently some carbazole antibiotics such as carazostatin, neocarazostatin and antiostatin bearing the free phenolic hydroxy group have been reported as free radical scavenger[9,11] or antioxidative agent.[12] Thus, the 5-LPO inhibitory activity of epocarbazolins are probably due to radical scavenger activity.

Experimental

General

The IR and UV spectra were determined on a JASCO IR-810 and a UVIDEC-610C spectrometer, respectively. The ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JMN-GX400. MS spectra were obtained with a JEOL JMS-AX505H mass spectrometer.

5-LPO Inhibitory Activity[10]

RBL-1 cells were grown for 5 days at 37°C and 5% CO_2 in RPMI1640 with 20% heat-inactivated calf serum. After harvested by centrifugation and washed twice with DULBECCO's phosphate-buffered saline without calcium and magnesium (PBS(−)) containing 1 mM EDTA, the cells were sonicated in the same buffer. The suspension was centrifuged at $13,000 \times g$ and the supernatant was stored at −70°C as enzyme solution. Enzyme assays were conducted by diluting the enzyme solution to desired specific activity with PBS(−) containing 28.5 mM phosphate, 1 mM EDTA, 0.9 mM ATP and 0.9 mM glutathione. The enzyme dilution (110 μl) was preincubated at 37°C for 5 minutes, and then mixed with a test sample in 20 μl of 10% DMSO-PBS(−) in the microplate. The reaction was started by addition of 5 μl of substrate (2 mM arachidonic acid plus 25 mM calcium chloride in ethanol-water (3:1)). After incubation for 5 minutes the reaction was terminated by addition of 135 μl of ethanol and the solution was centrifuged. The supernatant was analyzed for 5-HETE by liquid chromatography (Rainin Dynamax C-18, i.d. 4.6 × 50 mm) with HPLC solvent (85% methanol-29.2 mM lithium acetate buffer pH 6.3) at 1 ml/minute. Detection was done at 230 nm with a Waters 481 detector and the peak area integration with

a Hewlett Packard 3396-A integrator.

Methylation of Epocarbazolin B

To a mixture of NaH (450 mg of 60% oil suspension) and DMF (10 ml), epocarbazolin B (120 mg) and CH_3I (200 μl) were added and stirred for 2 hours at room temperature. The reaction mixture was poured into water (100 ml) and the *N,O*-methylated product was extracted with ethyl acetate. The solvent extract was concentrated to dryness *in vacuo* and the residue was subjected to reversed phase silica gel column chromatography. Development with acetonitrile-water (9:1, v/v) gave tetramethylepocarbazolin B (55.9 mg). FAB-MS (positive): *m/z* 439 (M^+); IR (KBr) 2930, 1610, 1460, 1420, 1235, 1090 cm^{-1}.

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