MLR-52, (4'-DEMETHYLAMINO-4',5'-DIHYDROXYSTAUROSPORINE), A NEW INHIBITOR OF PROTEIN KINASE C WITH IMMUNOSUPPRESSIVE ACTIVITY

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In the course of screening with the mixed lymphocyte reaction, a new inhibitor of protein kinase C with immunosuppressive activity was isolated from the fermentation broth and mycelia of *Streptomyces* sp. AB 1869R-359. Although certain similarities exist, this strain is morphologically and physiologically distinct from other reported producers of staurosporine-related compounds. We have found that this strain produces relatively high levels of staurosporine and the new minor compound MLR-52, which possesses the indolo[2,3-*a*]carbazole chromophore of staurosporine, but differs in the substitution pattern of the sugar moiety. Their structures have been elucidated by mass and NMR spectra. MLR-52 has been shown to inhibit the enzymatic activity of protein kinase C and the murine mixed lymphocyte reaction.

Staurosporine was originally discovered by ŌMURA and shown to have modest *in vitro* antifungal activity and strong hypotensive activity.[1] Like many other antibiotics, staurosporine was rediscovered several years later in a mechanistic-based screen. In 1979, NISHIZUKA and colleagues described a phospholipid/Ca^{++} dependent protein kinase (protein kinase C) that was directly activated by diacylglycerol.[2] This enzyme was subsequently shown to be the predominant cellular receptor for phorbol esters, and a central role for protein kinase C in signal transduction has been described.[3] Following binding of a large class of hormones and other cellular effectors to their individual receptors, phospholipase C is activated resulting in production of inositol 1,4,5-trisphosphate and diacylglycerol from phosphatidylinositol 4,5-bisphosphate.[4] Diacylglycerols, like phorbol esters, activate protein kinase C which modulates other signal transduction cascades and stimulates cell division during activation of T cells and other model systems.[5] Based on the importance of this enzyme for signal transduction and the control of cellular proliferation, a search for agents that inhibit protein kinase C was undertaken. The first potent inhibitor identified was staurosporine,[6] which has been found to be a nonspecific inhibitor of protein kinases.[7] Since the protein kinases are a large family of enzymes that mediate the response of eukaryotic cells to a wide variety of external stimuli,[8] it is not surprising that staurosporine has many biological effects. For example, staurosporine activates macrophages,[9] inhibits the neutrophil respiratory burst,[10] blocks the proliferative response of T lymphoblasts to mitogens[11] and is markedly cytotoxic.[6]

Materials and Methods

Microorganism

The microorganism that produces MLR-52 was isolated from a soil sample collected near Dorado, Puerto Rico. The culture is a *Streptomyces* species which we have designated strain AB 1869R-359. A subculture was deposited with the National Center for Agricultural Utilization Research, United States

Department of Agriculture, 1815 North University Street, Peoria, Illinois 61604, U.S.A. It was assigned the accession number NRRL B-16735.

Culture Characterization

Most of the cultural and physiological characteristics of strain AB 1869R-359 were examined using the methods and media described by SHIRLING and GOTTLIEB,[12] WAKSMAN[13] and GORDON *et al.*[14] Incubation for cultural characteristics and carbon utilization was at 28°C for 21 days. The technique of KORN-WENDISCH and KUTZNER[15] was used to observe reduction of nitrate. Analysis of the whole-cell diaminopimelic acid isomer was done by the method of BECKER *et al.*[16]

Fermentation

MLR-52 was produced by fermentation in a 42-liter stirred fermentor (LH Fermentation). The fermentor was charged with 30 liters of a medium consisting of glucose monohydrate 2%, molasses 0.5%, F-152 liquid peptone (Inolex) 1%, primary yeast (Universal Foods) 0.5% and $CaCO_3$ 0.2%. The medium was prepared in distilled water and the pH was not adjusted. Sterilization was at 121°C and 1.05 kg/cm^2 for 1 hour. Inoculum for the fermentation was prepared in 2-liter Erlenmeyer flasks containing 600 ml of a medium consisting of glucose monohydrate 1.5%, soy flour 1.5%, yeast extract (Difco) 0.1%, NaCl 0.1% and $CaCO_3$ 0.1% in distilled water. The flasks were seeded at 0.5% with vegetative mycelium form previous inoculum which had been maintained at −70°C. Incubation of the seed flasks was at 28°C for 72 hours on a rotary shaker operating at 225 rpm (5.08 cm stroke). The resulting growth was used at 5% to inoculate the fermentor. During fermentation the temperature was controlled at 28°C. Agitation was 250 rpm, the air flow was 0.7 vol/vol/minute and the head pressure was maintained at 0.35 kg/cm^2. Foam was controlled with a silicone antifoam, XFO 371 (Ivanhoe Industries), added initially at 0.01% and then available on demand.

Chemistry

Optical rotations were measured on a Perkin-Elmer Model 241 polarimeter in a 10 cm cell. Melting points were determined on a Hoover Unimelt and are reported uncorrected. Mass spectra were measured on a Kratos MS-50 mass spectrometer. Ultraviolet spectra were recorded on a Perkin-Elmer Lambda 3B UV-visible spectrophotometer and infrared spectra on a Nicolet model 60SX FT-IR attached to a Nicolet computer. NMR spectra were acquired on a General Electric GN500 spectrometer. ^{13}C and ^{1}H NMR spectral data for staurosporine and MLR-52 are reported in tables within the text.

Murine MLR

The immunosuppressive activity of compounds was assessed with two-way mixed lymphocyte reactions as previously described.[17] Briefly, BALB/c and C57BL/6 mice (female, 17∼18 g, 36∼42 days old) were sacrificed and spleens were aseptically removed. Spleens were homogenized and contaminating erythrocytes were lysed by suspension in EDTA - ammonium chloride solution. The BALB/c and C57BL/6 spleen cell suspensions were diluted to 2.5×10^6 viable cells/ml and pooled, before mixed cultures (200 µl/well) were established in 96-well microtiter plates. After 72-hours incubations at 37°C in a humidified atmosphere of 5% CO_2 in air, increases in proliferation were assessed by cellular uptake of tritiated thymidine. The IC_{50} of immunosuppressive compounds was graphically determined after incorporation of [*methyl*-^{3}H]thymidine was corrected for incorporation in unstimulated cultures and normalized as a percent of incorporation in stimulated cultures.

Inhibition of Protein Kinase C

The ability of MLR-52 to inhibit protein kinase C was determined with PepTag reagents obtained from Promega (U.S.A.). Reactions (25 µl) containing 25 ng of purified protein kinase C in 20 mM HEPES, pH 7.4, 1.3 mM $CaCl_2$, 1.0 mM DTT, 10 mM $MgCl_2$, 1 mM ATP and 0.2 mg/ml phosphatidyl serine were incubated for 30 minutes at 30°C. Reactions were stopped in a boiling water bath, 1 µl of 80% glycerol was added to each tube, and samples were transferred to wells of a 0.8% agarose gel in 50 mM Tris-HCl, pH 8.0. The current was applied immediately, and after 30 minutes at 150 volts the gel was photographed under UV illumination. Conversion of peptide PepTag C1 to the phosphorylated product was quantitated

with a LKB 2202 Ultroscan Laser densitometer, and the peak heights were normalized as a percent of the conversion resulting from protein kinase C observed in the absence of MLR-52.

Results

Comparison of Strains

The culture that produces MLR-52 is clearly a *Streptomyces* species; whole-cell hydrolysates of the culture contain major amounts of the LL isomer of diaminopimelic acid, and it exhibits typical *Streptomyces* morphology. Table 1 compares some morphological and physiological characteristics of strain AB 1869R-359 to other *Streptomyces* species that make staurosporine and related compounds. Table 2 shows the carbon utilization patterns of the same cultures. Although strain AB 1869R-359 has a gray-colored mature spore mass and a smooth spore surface, it is different from other cultures in the tables with these features based on spore chain morphology, physiological test reactions and carbon source utilization pattern.[18~23]

Fermentation

The harvest pH of the 7-day fermentation was 7.9. Growth was visually evaluated as moderate, reflecting the limited nutrients in the medium. Approximate production yields were calculated from the weight of the isolated compounds, indicating 0.3 mg/liter for MLR-52 and 100 mg/liter for staurosporine.

Table 1. Morphological and physiological features of *Streptomyces* sp. reported to produce staurosporine-related compounds.

Strain	Spore mass color	Spore surface	Spore chain	Melanoid pigment	
				ISP-6	ISP-7
Streptomyces sp. AB 1869R-359	Gy	SM	RA-S	+	−
S. sp. RK-286	Gy	SM	RA	+	+
S. hygroscopicus ATCC 57330	Gy	SM	S	−	−
S. actuosus	Gy	SM	RF	+	+
S. sp. N-71	Gy	SM	S		
S. sp. N-115	R, W	SP	S		
S. sp. N-126	Gy, W	SM	RA-S	+	+
S. sp. N-128	R, W	SP	RA-S		
S. sp. N-139	Gy, W, Y	SM	RF	−	−
S. sp. C-71799	Gy	SM	RF	−	−
S. platensis subsp. *malvinus* RK-1409	Gy, W	SM	S	−	−

Strain	Gelatin liquefaction	Nitrate reduction	Milk peptonization	Soluble pigment	Ref
Streptomyces sp. AB 1869R-359	+	+	−	+	
S. sp. RK-286				−	18
S. hygroscopicus ATCC 57330	+	−	+	+	19
S. autuosus	+	+	+	−	20, 21
S. sp. N-71	+		+	+	22
S. sp. N-115	−		+	+	22
S. sp. N-126	−		+	+	22
S. sp. N-128	−		+	+	22
S. sp. N-139	−		+	−	22
S. sp. C-71799	−	+	−	+	9
S. platensis subsp. *malvinus* RK-1409				+	23

Gy, Gray; R, red; W, white; SM, smooth; SP, spiny; RA, open looped; S, spiral; RF, straight to flexuous.

Table 2. Carbon source utilization patterns of *Streptomyces* sp. producing staurosporine and related compounds.

Strain	L-Arabinose	D-Xylose	*m*-Inositol	D-Mannitol	D-Fructose
Streptomyces sp. AB 1869R-359	−	+	+	−	+
S. sp. RK-286	−	−	+	+	−
S. hygroscopicus ATCC 57330			−		
S. actuosus	+	+	+	+	+
S. sp. N-71	−	−	−	−	−
S. sp. N-115	+	+	+	+	+
S. sp. N-126	+	+	+	+	+
S. sp. N-128	+	+	+	+	+
S. sp. N-139	+	+	−	−	−
S. sp. C-71799	+	+	+	+	−
S. platensis subsp. *malvinus* RK-1409	−	+	+		+

Strain	L-Rhamnose	Sucrose	Raffinose	Ref
Streptomyces sp. AB 1869R-359	−	−	−	
S. sp. RK-286	+	+	+	18
S. hygroscopicus ATCC 57330				19
S. actuosus	+	+	+	21
S. sp. N-71	−	−	−	22
S. sp. N-115	+	+	+	22
S. sp. 126	+	+	+	22
S. sp. N-128	+	+	+	22
S. sp. N-139	+	−	−	22
S. sp. C-71799	+	+	+	9
S. platensis subsp. *malvinus* RK-1409	+	+	+	23

Isolation

In a search for immunomodulators, extracts of microbial fermentations were tested for ability to inhibit the mitogenic response observed in mixed murine splenocyte cultures. This assay was used to guide the fractionation of a fermentation broth of *Streptomyces* sp. AB 1869R-359 as follows. To 25 liters of whole broth was added 12 liters of acetone, the mixture was stirred for 1.5 hours and then extracted with ethyl acetate (2×12 liters). Combined ethyl acetate extracts were concentrated to an oil and then partitioned between chloroform-methanol-water (1 liter of each). The upper layer from this partition was concentrated to dryness and triturated with hexane (3×0.8 liters). The marc from this trituration was partitioned between ethyl acetate-ethanol-water (3:1:2) and the upper layer was concentrated to an oily solid. This solid was chromatographed over a Sephadex LH-20 column developed with methanol. Fractions that inhibited the mixed lymphocyte reaction from the LH-20 column were combined based upon their behavior on TLC to yield 2.63 g staurosporine (**2**), and a second active band. The second band was subjected to countercurrent chromatography on an Ito multi-layered coil planet centrifuge in a solvent system of acetone-ethyl acetate-methanol-water (2:1:1:1) with the lower phase stationary. Immunosuppressive fractions from this countercurrent chromatography were combined and concentrated to yield 7 mg of pure MLR-52 (**1**).

Physico-chemical Properties

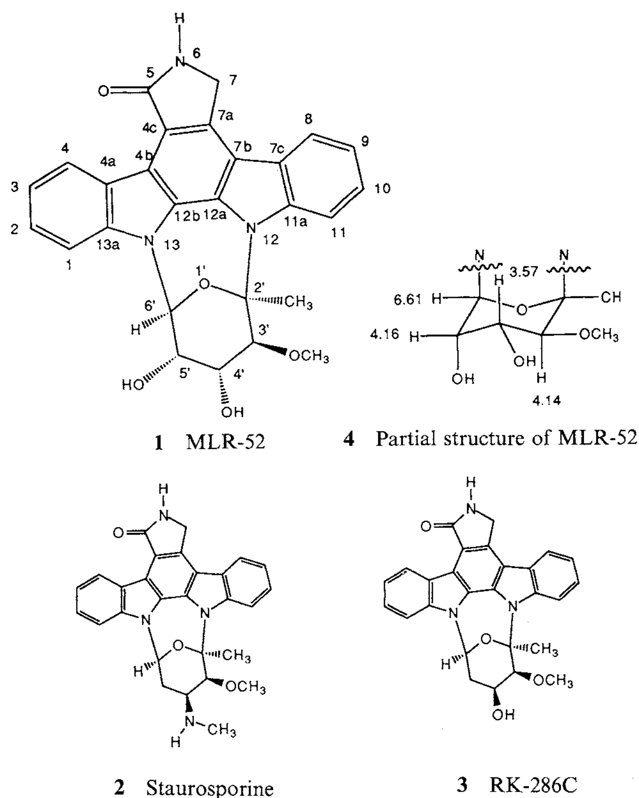
MLR-52, $[\alpha]_D$ 68° (*c* 0.093, MeOH), mp 263～268°C, had Rf values on Merck silica gel TLC plates of 0.27 in EtOAc (0.07 for staurosporine), 0.09 in $CHCl_3$-MeOH (95:5) (0.17 for staurosporine) and 0.24 in toluene-*i*-PrOH (8:2) (0.45 for staurosporine). An ultraviolet spectrum obtained in methanol

contained peaks at λ_{max} 368 nm (ε 22,600), 351 (21,500), 332 (30,000), 317 (25,000), 286 (72,100), 234 (48,500) and 206 (48.500). These values were unchanged upon addition of acid or base. An infrared spectrum (KBr pellet) contained bands at 3435, 2922, 1635, 1590, 1455, 1420, 1395, 1370, 1350, 1340, 1320, 1275, 1225, 1200, 1125, 1100, 1055 and 1005 cm^{-1}.

Structure Determination

A high resolution positive ion fast atom bombardment mass spectrum of **1** gave an exact mass of *m/z* 469.1638 (M+H exact mass 470.1716) consistent with a molecular formula for **1** of $C_{27}H_{23}N_3O_5$. ^{13}C NMR and DEPT[24] spectra indicated 27 unique carbon atoms (see Table 3). A COSY experiment defined two isolated ABCD aromatic spin systems which could be expanded upon *via* a heteronuclear multiple-bond correlation (HMBC)[25] map (see Table 3) to define the indolocarbazole structure (for atoms 1~13a) of staurosporine.[26] Remaining signals defined an attached sugar moiety structurally related to that in the staurosporine derivative RK-286C (**3**).[27] The stereochemistry for this fragment (**4**) was defined by an analysis of the coupling constants and single frequency proton decoupling experiments as follows: An *axial-axial* coupling constant of 10.3 Hz between proton signals for 3′ and 4′ at δ 4.14 (d, 1H, J=10.3 Hz) and δ 3.57 (dd, 1H, J=10.3 Hz), respectively, indicated that the 3′ methoxy and the 4′ hydroxy groups must each be *equatorial*. Further, the proton at 5′ (δ 4.16; dd, 1H, J=2.6, 1.8 Hz) must, as evidenced by the small coupling constant of 2.6 Hz to the 4′ hydrogen, be *equatorial* (*axial* hydroxyl at 5′). Of necessity, the 2′ methyl and 6′ proton are *equatorial*.

Fig. 1. Structures of MLR-52 (**1**), staurosporine (**2**), RK-286C (**3**) and partial structure of MLR-52 (**4**).



1 MLR-52

4 Partial structure of MLR-52

2 Staurosporine

3 RK-286C

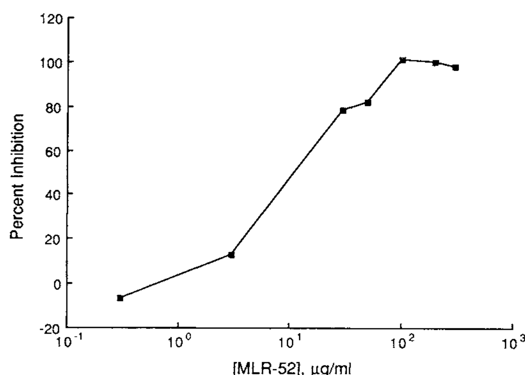
Table 3. ^1H and ^{13}C NMR assignments of MLR-52 (in DMSO-d_6).

Carbon No.	^{13}C NMR shift (multiplicity)	^1H NMR shift (multiplicity, J's)	Carbon No.	^{13}C NMR shift (multiplicity)	^1H NMR shift (multiplicity, J's)
1	108.7 (CH)	7.64 (br d, 1H, J=8.4 Hz)	9	120.1 (CH)	7.27 (br dd, 1H, J=7.7, 7.0 Hz)
2	125.5 (CH)	7.54 (br dd, 1H, J=8.4, 7.0 Hz)	10	124.8 (CH)	7.45 (br dd, 1H, J=8.8, 7.0 Hz)
3	119.7 (CH)	7.29 (br dd, 1H, J=8.1, 7.0 Hz)	11	115.5 (CH)	7.98 (br d, 1H, J=8.8 Hz)
4	125.8 (CH)	9.31 (br d, 1H, J=8.1 Hz)	11a	140.2 (Q)	
4a	122.8 (Q)		12a	127.8 (Q)	
4b	114.9 (Q)		12b	124.6 (Q)	
4c	119.2 (Q)		13a	136.4 (Q)	
5	171.8 (Q)		2'	95.6 (Q)	
7	45.4 (CH_2)	4.99 (d, 1H, J=17.9 Hz), 4.95 (d, 1H, J=17.9 Hz)	3'	83.1 (CH)	4.14 (d, 1H, J=10.3 Hz)
			4'	65.6 (CH)	3.57 (dd, 1H, J=10.3, 2.6 Hz)
7a	132.6 (Q)		5'	71.7 (CH)	4.16 (dd, 1H, J=2.6, 1.8 Hz)
7b	114.3 (Q)		6'	87.3 (CH)	6.61 (d, 1H, J=1.8 Hz)
7c	123.6 (Q)		2'-CH_3	29.0 (CH_3)	2.38 (s, 3H)
8	120.9 (CH)	8.01 (br d, 1H, J=7.7 Hz)	3'-OCH_3	61.6 (CH_3)	3.62 (s, 3H)

Table 4. *In vitro* immunosuppressive activity.

Agent	Mouse mixed lymphocyte reaction IC_{50}, nM
MLR-52	1.9±0.2
Staurosporine	1.3±0.2
FK-506	0.39±0.12
Cyclosporin	2.5±0.8

Fig. 2. Inhibition of protein kinase C by MLR-52.



Biological Activities

The *in vitro* immunosuppressive activities of MLR-52 and staurosporine were compared to that of clinically useful immunosuppressants. As shown in Table 4, MLR-52 was nearly as potent as staurosporine in inhibiting the mitogenic response in mixed lymphocyte cultures, and both MLR-52 and staurosporine show *in vitro* immunosuppressive potency similar to FK-506 or cyclosporine. As shown in Fig. 2, MLR-52 inhibits protein kinase C phosphorylation of a small tyrosine containing peptide.

Discussion

Bioassay-guided fractionation of a fermentation broth of *Streptomyces* sp. AB 1869R-359 resulted in the identification of staurosporine and the new minor compound MLR-52. MLR-52 has the same indolo[2,3-*a*]carbazole chromophore of staurosporine, but differs in the substitution pattern of the sugar moiety. Staurosporine was first isolated from an microorganism originally characterized as *Streptomyces staurosporeus*[1] but recently reclassified as a *Saccharothrix* sp.[28] Since the initial report, staurosporine and staurosporine-related compounds have been found in the culture broths of many actinomycetes. It is interesting, however, that all of the producing cultures fall into two taxonomic group. Most of the producers are *Streptomyces*, but other producing genera include *Actinomadura*,[29,30] *Nocardiopsis*[9,31] and *Saccharothrix*[32] species. The latter three genera all possess a cell wall III chemotype and are thought to be related.[33,34]

Staurosporine was first identified as a weakly active antibacterial and antifungal agent,[1] but was subsequently shown to be a potent cytotoxic agent and to inhibit the activity of a variety of protein kinases

in vitro.[7] Since the discovery of staurosporine, several other microbial products have been identified that inhibit protein kinases that contain the same indolo[2,3-*a*]carbazole chromophore of staurosporine.[7] Changes in the sugar moiety have been previously shown to not alter ability to inhibit protein kinase C. In fact, the aglycone of K-252a retains biological activity[35] suggesting that the indole carbazole system, which is identical in MLR-52 and staurosporine, is predominantly responsible for interaction with protein kinase C. Since the mitogenic response of T cells is known to be mediated in part by activation of protein kinase C,[36] it is not surprising that a new staurosporine-related compound shows potent *in vitro* immunosuppressive activity.

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