OLIGOMYCIN F, A NEW IMMUNOSUPPRESSIVE HOMOLOGUE OF OLIGOMYCIN A

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Oligomycin F, a new homologue of oligomycin A (**1**), was isolated from a *Streptomyces* species and structure **2** was deduced by NMR methods. Compound **2** is highly active against plant pathogenic fungi and is an extremely potent suppressive agent for various immunological systems.

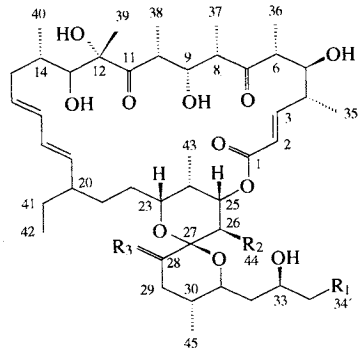
Results and Discussion

The *Streptomyces* sp. strain No. A 171 was isolated from a soil sample collected in Göttingen, Germany and was conspicuous in a screening for biological control of soil borne plant pathogenic fungi.

Culture media and extracts of strain A 171 exhibited strikingly high activity against barley powdery mildew (*Erysiphe graminis* DC f. sp. *hordei* Marchal) and other plant pathogenic fungi. The antifungal effect is caused by a mixture of polar substances which was enriched by repeated adsorption on Amberlite XAD-2 followed by gradient elution and chromatography on silica gel (Fig. 4). The mixture was separated into two major components by column chromatography.

Component 1 was separated into two fractions by preparative HPLC. By its molecular weight, IR spectra, by different NMR techniques, its antibiotic activity spectra, and by chromatographic comparison (HPLC) with an authentic sample*, one of these fractions was identified as oligomycin A[1~4] (**1**).

The NMR data of the second fraction are very similar to those of oligomycin A (**1**) but differ by an additional methylene group, as the molecular weight (14 amu more) indicates. The common appearance, the similarity of the NMR spectra and the chromatographic behaviour has led us to the assumption that A 171-1 is a homologue of oligomycin A (**1**).



Oligomycin A (**1**)	$R_1=H$	$R_2=CH_3$	$R_3=H_2$
Oligomycin F (**2**)	$R_1=CH_3$	$R_2=CH_3$	$R_3=H_2$
NK 130119 (**3**)	$R_1=H$	$R_2=CH_2CH_3$	$R_3=H_2$
NK 86-0279-I (**4**)	$R_1=H$	$R_2=CH_2CH_3$	$R_3=O$
Oligomycin B (**5**)	$R_1=H$	$R_2=CH_3$	$R_3=O$

* Fluka BioChemika No. 75351 (oligomycin A), 75354 (B) and 75352 (mixture A, B, C).

Table 1. Complete NMR data of **2** and related compounds.

C-No.	Oligomycin F ($CDCl_3$) experimental				Oligomycin A (CD_3OD) literature[2]	Oligomycin A ($CDCl_3$) experimental	NK 130119 ($CDCl_3$) literature[6]
	δ (C) ppm	δ (H) ppm		J (Hz)	δ (C) ppm	δ (C) ppm	δ (C) ppm
1	165.12 O–C=O				166.80 C=O	165.14 C=O	164.98 C=O
2	122.78 CH sp^2	5.83	dd	15.5, 0.7	123.55 CH sp^2	122.77 CH sp^2	122.82 CH sp^2
3	148.38 CH sp^2	6.65	dd	15.5, 10.0	150.66 CH sp^2	148.41 CH sp^2	148.55 CH sp^2
4	40.17 CH	2.40	dd	~ 5	42.63	40.18 CH	40.25
5	73.02 CH–O	3.79	d	~10	74.20 CH	73.03 CH	73.01
6	46.61 CH	2.73	dq	7.3, 1.3	45.76 CH	46.59 CH	46.67
7	220.35[a] >C=O				218.76 C=O	220.33 C=O	219.97 C=O
8	41.96[a] CH	3.62	dq	8.7, 6.8	48.95	41.96 CH	45.71
9	72.65[a] CH–O	3.97	m		74.26 CH	72.67 CH	72.64
10	45.70 CH	2.78	dq	6.9, 3.0	43.73 CH	45.72 CH	41.95
11	220.01[a] >C=O				222.99 C=O	220.01 C=O	220.35 C=O
12	83.05 C_{quart}–O(H)				84.24 C_{quart}	83.05 C_{quart}	83.02 C_{quart}
13	72.23 CH–O	3.96	m		74.30 CH	72.25 CH	72.26
14	33.54 CH	1.92	dt	9.5, 4.5	35.21	33.54 CH	33.54
15	38.48 CH_2	2.21, 1.96	dm	14.3	39.60	38.48 CH_2	38.46
16	129.39 CH sp^2	5.46	ddd	15.0, 10.5, 3.5	131.49 CH sp^2	129.41 CH sp^2	129.41 CH sp^2
17	132.45 CH sp^2	6.04	ddd	15.0, 10.5, 1.8	133.93 CH sp^2	132.45 CH sp^2	132.43 CH sp^2
18	130.32 CH sp^2	5.94	dd	15.0, 10.5	132.63 CH sp^2	130.32 CH sp^2	130.31 CH sp^2
19	137.85 CH sp^2	5.26	dd	15.0, 9.7	138.07 CH sp^2	137.86 CH sp^2	137.76 CH sp^2
20	46.07 CH	1.86	dm	~ 3	39.06 CH	46.08 CH	46.17
21	31.52 CH_2	1.25, 1.41			32.68	31.50 CH_2	31.55
22	31.03 CH_2	1.55, 1.49			32.49	31.02 CH_2	31.02
23	69.08 CH–O	3.795	dt	11.5, 2.5	69.74 CH	69.09 CH	69.08
24	35.87 CH	2.15	dd		37.59	35.88 CH	36.06
25	76.26 CH–O	4.95	dd	11.5, 5.0	77.65 CH	76.22 CH	76.33
26	37.76 CH	1.83	dq	11.5, 6.3	39.06	37.76 CH	44.15
27	99.23 O–C_{quart}–O				100.28 C_{quart}	99.25 C_{quart}	100.13 C_{quart}
28	26.08 CH_2	1.96, 1.25	d d		27.01	26.03 CH_2	26.36
29	26.57 CH_2	2.12, 1.41	d d		27.69	26.56 CH_2	26.53
30	30.66 CH	1.59			31.76	30.55 CH	30.52
31	67.26 CH–O	4.02	dt	10.5, 2.5	68.58 CH	67.28 CH	67.28
32	40.30 CH_2	1.66, 1.25	dd		43.63	42.65 CH_2	42.66
33	69.75 CH–O	3.75			65.09 CH	64.71 CH	64.70
34	31.18 CH_2	1.49, 1.41	d		25.10	24.83 CH_3	24.81
34′	9.76 CH_3	0.97	t	7.0	—	—	—
35	17.97 CH_3	1.19	d	6.6	18.40	17.97 CH_3	17.92
36	8.31[a] CH_3	1.08	d	7.3	9.55	8.32 CH_3	9.35
37	14.14[a] CH_3	1.12	d	6.8	8.81	14.13 CH_3	8.31
38	9.30[a] CH_3	1.04	d	6.9	15.24	9.29 CH_3	14.11
39	21.00 CH_3	1.14	s		22.59	21.02 CH_3	21.02
40	14.52 CH_3	1.01	d	6.7	15.15	14.52 CH_3	14.51
41	28.57 CH_2	1.38, 1.30	dq	d	29.94	28.56 CH_2	28.58
42	12.12 CH_3	0.83	t	7.5	12.55	12.12 CH_3	12.09
43	6.12 CH_3	0.85	d	6.9	7.32	6.11 CH_3	6.10
44	11.85 CH_3	0.98	d	6.3	12.19	11.82 CH_3	21.61
44′	—				—	—	14.37 CH_3
45	11.31 CH_3	0.92	d	7.0	11.59	11.26 CH_3	11.46

Five OH signals at δ ~3.3, ~3.5, 3.7~3.8, 2 × ~4.0.

[a] Signal assignments different from those of YAMAZAKI *et al.*[6]

A further macrolide corresponding to **1** is NK 130119[5] (identical with NK 86-0279-II[6], **3**) which was described recently but is clearly different from the macrolide A 171-1 by the ^{13}C NMR data (see Table 1). The same is valid for NK 86-0279[7] (identical with NK 86-0279-I[6], **4**), another derivative of oligomycin A with an additional methylene group and a fourth carbonyl group.

In spite of the differences in solvents used, it is possible to correlate the ^{13}C NMR data of **1** obtained in deuteriomethanol[2] with those of **1** and A 171-1 measured in deuteriochloroform due to their shift and APT differentiation. A very close similarity results for **1**, **3** and A 171-1 with respect to the signals concerning the macrolactone partial structure. Thus, the differences must be due to the spirosystem. The ^{13}C NMR data of C-23 to C-31 are corresponding in all three structures with exception of C-26 in NK 130119 (**3**), however data differ with regard to the side chain (C-32 to C-34).

Evaluation of the ^{1}H-, ^{13}C- and DEPT-NMR spectra by chemical shift arguments reveals six Csp ^{2}H-groups (and therefore three double bonds), two carbonyl groups, and one ester group. Comparison of the spectra obtained at different temperatures and concentrations, as well as COLOC spectra, show five OH-groups. As for oligomycin A (**1**) two further C_{quart} (one C–O and one O–C–O), seven CH–O– (forming four CH–OH-groups), nine CH–, eight CH_2-structure elements (one more as in **1**), and eleven CH_3-groups were found.

A differentiation of the signals above $\delta = 1.15$ is difficult in the one-dimensional ^{1}H NMR spectrum owing to extensive overlap. However, the two-dimensional C, H correlation spectrum (HMQC) easily differentiates the methyl signals into a singlet, two triplets and nine doublets. Our results are consistent with the formula $C_{46}H_{76}O_{11}$, and the remaining three double bond equivalents correspond to a tricyclic system as in **1**.

C, H signals were assigned largely by an inverse C, H correlation experiment. Difficulties arose only at C-21, C-22 and C-34 methylene groups, due to overlap and bad resolution. The H, H-COSY spectrum shows a coupling of the C-34′ methyl protons ($\delta_C = 9.76$, $\delta_H = 0.97$) with one of these methylene groups. As substitution of the ring methylene groups C-21, C-22 would result in a further methine group (which was not found), C-34 and C-34′ must form a terminal ethyl group. Undoubtedly, this leads to the structure fragments which are drawn bold in Fig. 1; data are given in Table 1.

The complete connectivity was obtained by inverse COLOC spectra (HMBC). Aside from some minor differences in signal assignment (see Table 1), our data agree very well with those of YAMAZAKI

Fig. 1. Structure of **2** (A 171-1) with structure fragments (drawn bold) by H-H COSY.

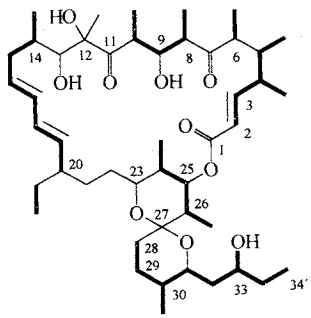
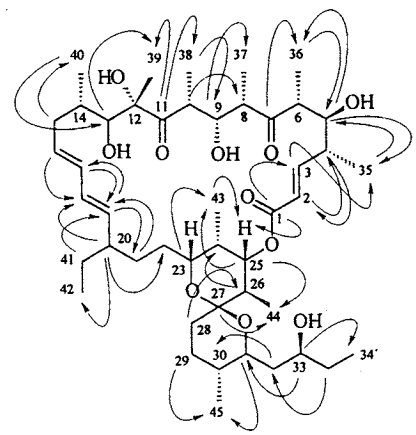


Fig. 2. Inverse COLOC-$^{3}J_{C-H}$ couplings of **2** (A 171-1), arrows are directing from C to H (for ^{2}J and ^{4}J couplings see experimental part).



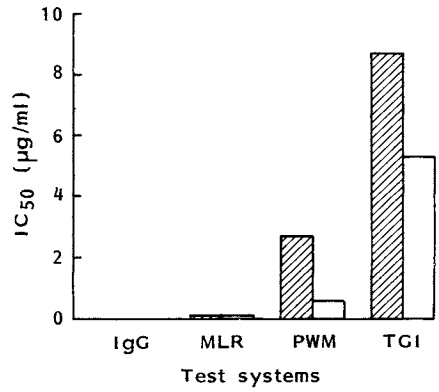
et al.[6], leading to structure **2** by the pattern of ^3J-couplings (Fig. 2).

The terminal methyl group of oligomycin A (**1**) and NK 130119 (**3**) ($\delta = 24.83$) is substituted in **2** by an additional methylene ($\delta_{C\text{-}34} = 31.18$) and a methyl group ($\delta_{C\text{-}34'} = 9.76$). The new antibiotic for which we suggest the name oligomycin F thus is 33-demethyl-33-ethyl-oligomycin A. The configuration of the stereo centers was not analyzed in detail. However, the considerable similarity of the NMR spectra suggests that it is the same as in oligomycin A[2] (**1**) and oligomycin B[8] (**5**).

The proliferation of human peripheral blood lymphocytes in a mixed lymphocyte reaction (MLR), after pokeweed mitogen stimulation (PWM), or the IgG production of human spleen cells (IgG), in the presence of various concentrations of oligomycin A and F has been determined. The proliferation of a mouse Meth A sarcoma cell line (TGI) was also tested to exclude cytotoxic effects. Results obtained show (Fig. 3) that in a concentration of 0.1 μg/ml the two oligomycines exhibit a 50% suppression of the mixed lymphocyte reaction. A 6- to 26-fold higher concentration of the two oligomycins is needed to induce a 50% suppression of the PWM induced proliferation. However, a 10^6-fold lower concentration of oligomycin A or F is sufficient to suppress the IgG production of human spleen cells to 50%. In conclusion, the data indicate that the two oligomycins are potent suppressive agents for human B-cell activation.

Fig. 3. Activity of oligomycins in immunological systems.

Closed: oligomycin A (**1**), open: oligomycin F (**2**).



	IC_{50} (μg/ml)			
	IgG	MLR	PWM	TGI
Oligomycin A	0.00000010	0.10	2.680	8.70
Oligomycin F	0.00000010	0.10	0.620	5.30

Experimental

IR spectra: Perkin-Elmer, model 297; (KBr). ^1H and ^{13}C NMR spectra, Varian VXR 500, Bruker AMX 300 (TMS as internal standard). MS: FD (field desorption); Finnigan 8200. UV spectra: Beckman DB-G. TLC: Polygram SIL G/UV_{254}; Macherey-Nagel and Co. Hydrophobic interaction chromatography: XAD-2, 0.3~1.0 mm; Serva, Heidelberg. HPLC: spectral-digital-photometer A0293 with two pumps type 64 A0307 and HPLC software V2.12 (Knauer, Berlin); analytical separations: pre column 4×4 mm and column 4×250 mm (vertex) with Europrep RP 60-10 C-18 60 Å 7~12 μm (Eurochrom, Berlin) or Lichrosorb RP C-18 7 μm (E. Merck, Darmstadt); preparative separations: pre column 16×30 mm and column 16×250 mm (vertex) with Europrep RP 60-10 C-18 60 Å 7~12 μm (Eurochrom, Berlin).

Streptomyces sp. A 171

The strain No. A 171 was isolated from a German soil sample using the modified procedure of HERR[9]. In the first screening steps, the plant pathogenic fungus *Fusarium culmorum* was used as test organism. Strain A 171 exhibited strong activity against several plant pathogenic fungi *in vitro* and a strikingly high activity against powdery mildew of barley *ad planta*.

Cultural characteristics were observed on the media described by SHIRLING and GOTTLIEB[10] and WAKSMAN[11]. Incubation was carried out at 25°C for 14 to 21 days. Morphological observations were made by light microscopy of cultures grown at 25°C for 14 to 21 days on inorganic salts-starch agar and

Table 2. Macroscopic characteristics of *Streptomyces* sp. A 171. Observations were made after 14 to 21 days incubation at 25°C.

Culture media	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Sucrose nitrate agar (CDA)	Moderate	—	Pale yellow	Yellow
Yeast extract-malt extract agar (ISP No. 2)	Moderate	—	Yellow	Yellow
Oat meal agar (ISP No. 3)	Moderate	Pink	Pale yellow	—
Inorganic salts starch agar (ISP No. 4)	Strong	Dark gray	Light gray	—
Glycerine asparagine agar (ISP No. 5)	Weak	—	White	—
Peptone yeast extract iron agar (ISP No. 6)	Moderate	—	Pale yellow	Intensive yellow
Potato dextrose agar (PDA)	Moderate	—	Pale yellow	—
Tryptic soy agar (TSA)	Moderate	—	Pale yellow	Pale yellow
Soil extraction agar (SEA)	Weak	Pink	Pale yellow	—

soil extraction agar. Methods for physiological observations were those of SHIRLING and GOTTLIEB[10], and SMIBERT and KRIEG[12]. Utilization of carbon sources was examined by the method of PRIDHAM *et al.*[13].

Table 3 (a) and (b); Physiological characteristics of A 171. Observations were made after 14 to 21 days incubation at 25°C.

(a)

Soluble pigments	Pale yellow
Melanin production on ISP No. 6	Grey ~ yellow
Nitrate reduction	−
Citrate utilisation	−
NaCl tolerance	3.0%
Starch hydrolysis (ISP No. 4)	+
Acid production from glucose	+
Catalase	+
Oxidase	+

(b)

Utilization of carbon sources	
L-Arabinose	−
Cellulose	−
D-Fructose	(−)
D-Glucose	+
i-Inositol	−
D-Mannitol	−
Raffinose	−
Rhamnose	−
Sucrose	+
D-Xylose	−

Fermentation

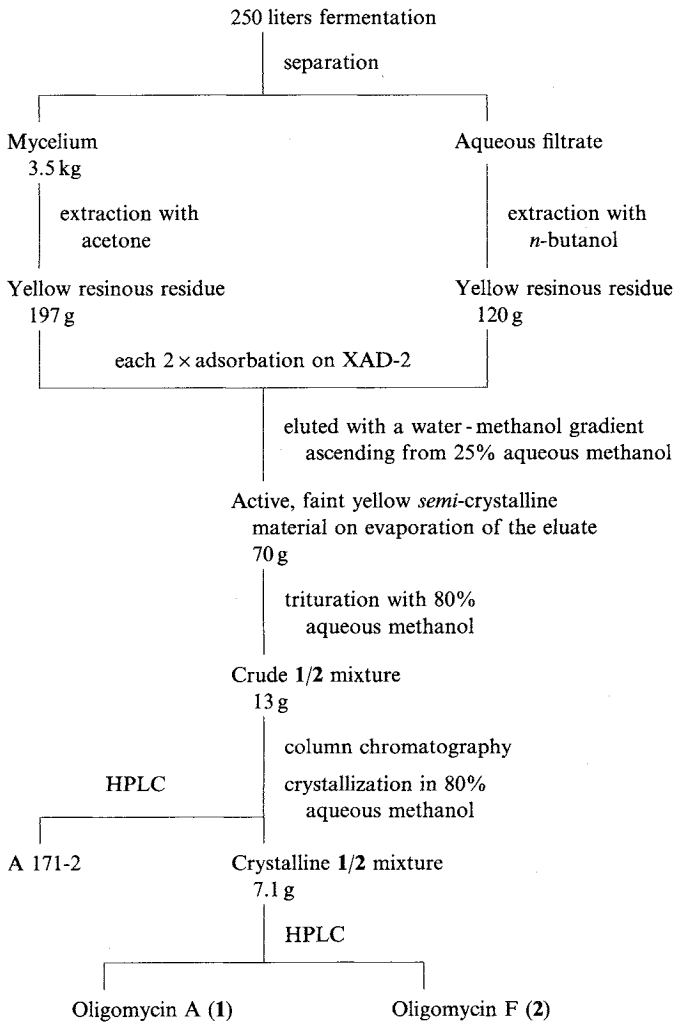
A loopful of *Streptomyces* sp. A 171 on mature slant culture in soil extraction agar was transferred to a 100-ml Erlenmeyer flask containing 50 ml of 2% sterile tryptic soy broth (TSB). The medium was adjusted to pH 6.5 prior to autoclaving. The flask was shaken on a rotary shaker (120 rpm, 2 cm throw) for 1 day at 25°C.

A 2.5 ml sample of seed culture was used to inoculate 250 ml of 2% tryptic soy broth in a 1-liter Erlenmeyer flask and was incubated as above for 24 hours. This culture was used to inoculate 7.0 liters 1% tryptic soy broth (pH 6.5) in a 10-liter jar fermentor and cultured at 30°C for 12 hours under aeration of 20 liters/minute with agitation of 400 rpm. The 7 liters culture was used to inoculate 250 liters production medium (TSB 1.0%, pH 7.0) in a 300-liter jar fermentor and this was cultured at 30°C for 36 hours under aeration of 5,000 liters/hour with an agitation of 250 rpm.

Isolation

Mycelium from the 250 liters fermentation was separated (3.5 kg wet weight) and extracted four times with each 10 liters acetone (Fig. 4). The aqueous filtrate was extracted three times with 60 liters *n*-butanol. The yellow resinous residues (197 g from acetone, 120 g from butanol) obtained on evaporation *in vacuo* (40°C) of the extracts each were suspended in 0.5 liter methanol, diluted with 3 liters water, and each were adsorbed on 3 liters XAD-2 resin (columns 7.5 × 65 cm). After washing with 2 liters water and 4.5 liters 25% methanol, the active substances were eluted with a water-methanol gradient ascending from 25% methanol. The active fractions were separated again in the same manner and combined, yielding 70 g of a faint yellow *semi*-crystalline material. On trituration with 80% aqueous methanol, 13 g crude mixture of **1** and **2**

Fig. 4. Isolation of oligomycin F (**2**).



remained undissolved. On column chromatography (column 75 × 80 cm, CH_2Cl_2-4% MeOH), 12 g of a **1/2** mixture was obtained which gave, on crystallization from 80% aqueous methanol, 7.1 g of **1/2** mixture. The mother liquid contained besides **1** and **2** a compound A 171-2 that moved more slowly in TLC (anisaldehyde-sulfuric acid: red-violet) and showed a molecular weight of 890 amu by FD-MS. This compound will be described later.

On analytical HPLC (isocratic 10% H_2O, 90% MeCN-H_2O aceotrope, flow rate 1.0 ml/minute, sample 1 μg/20 μl, detection at 225 nm), retention times were as following: oligomycin B[1] (**5**) (reference) 6.2 minutes, oligomycin A[1] (**1**) 8 minutes, oligomycin F (**2**) 9.8 minutes, oligomycin C[1] (**6**) (reference) 11.3 minutes. Separation of the **1/2** mixture on a preparative scale was done as follows: isocratic 10% H_2O, 90% MeCN-H_2O aceotrope, flow rate 8.0 ml/minute, sample 12 mg/400 μl, detection at 225 nm.

IR (KBr discs) λ_{max} (cm^{-1}) 3460~3480, 2970, 2935, 1704, 1647, 1462, 1387, 1290, 1228, 990. FD-MS *m/z* 805 (M+1). UV λ_{max}^{MeOH} (nm, qual.) 219 (sh), 239. ^1H NMR ($CDCl_3$, 499.8 MHz) and ^{13}C NMR ($CDCl_3$, 125.7 MHz) see Table 1. ^1H-^1H COSY NMR ($CDCl_3$, relay H pulse sequence, 499.84 MHz) 2→3, 3→4, 4→35, 4→5, 5→36 (very weak), 6→36, 8→37, 8→9, 9→10, 10→38, 14→40, 14→15 (δ=2.21, weak), 15 (δ=2.21)→15 (δ=1.96), 15 (δ=2.21, 1.96)→16, 16→17, 17→18, 18→19, 19→20, 42→41 (δ=1.30), 41 (δ=1.30)→21 (δ=1.41), 41 (δ=1.38)→22 (δ=1.55, very weak), 22 (δ=1.55)→23 (very weak), 24→43, 24→25, 25→26, 26→44, 28 (δ=1.25)→28 (δ=1.96), 28 (δ=1.25, 1.96)→29 (δ=1.41, 2.12), 29 (δ=1.41)→29

(δ=2.12), 29 (δ=1.41)→30, 30→45 (weak), 31→32 (δ=1.66), 32 (δ=1.66)→32 (δ=1.25), 32 (δ=1.25, 1.66)→33, 34 (δ=1.49)→34'.

HMBC NMR ($CDCl_3$, F1 300.13 MHz, F2 75.48 MHz; $^2J_{(C,H)}$ and $^4J_{(C,H)}$ couplings in addition to ^3J couplings of Fig. 2; numbers indicate atom numbers in **2**): $1^2J\rightarrow2$, $2^2J\rightarrow3$, $3^2J\rightarrow4$, $4^2J\rightarrow35$, $5^2J\rightarrow4$, $6^2J\rightarrow36$, $6^4J\rightarrow37$, $7^2J\rightarrow8$, $9^2J\rightarrow37$, $9^2J\rightarrow8$, $9^2J\rightarrow10$, $10^2J\rightarrow38$, $11^2J\rightarrow10$, $12^2J\rightarrow39$, $14^2J\rightarrow13$, $14^2J\rightarrow40$, 14^2J 15 both, $19^4J\rightarrow16$, $20^2J\rightarrow41$ both, $21^2J\rightarrow20$, $24^2J\rightarrow43$, $25^2J\rightarrow24$, $25^2J\rightarrow26$, $26^2J\rightarrow44$, $27^2J\rightarrow26$, $27^2J\rightarrow28$ (δ=1.96), $28^2J\rightarrow29$ both, $29^2J\rightarrow28$ both, $30^2J\rightarrow45$, $31^2J\rightarrow32$ both, $32^2J\rightarrow33$, $33^2J\rightarrow34$, $34^2J\rightarrow34'$, $38^2J\rightarrow10$, $41^2J\rightarrow42$, $42^2J\rightarrow41$ (δ=1.38), $43^2J\rightarrow24$.

$C_{46}H_{76}O_{11}$ (805.10) Calcd. C 68.63, H 9.51
Found: C 68.25, H 9.55

Preparation of Leukocytes

Fresh venous blood was drawn from healthy donors and anticoagulated with liquemin (La Roche, Switzerland). The blood was diluted with an equal volume of PBS without Ca^{2+} and Mg^{2+}. After depletion of platelets by centrifugation, the cell pellet was re-suspended to the original volume and the leukocytes (containing basophils) were isolated by density gradient centrifugation with lymphocyte separation medium (Boehringer Mannheim, Mannheim, FRG).

Mitogen Induced Proliferation Assay (LPT)

200 μl of cell suspension (1×10^6 cells/ml) in RPMI 1640 culture medium (supplemented with 10% heat inactivated fetal calf serum, 2 mM glutamine, 1% BME vitamins, 10 U/ml penicillin and 10 μg/ml streptomycin) were cultured with 2 μg/ml concanavalin A in flat bottomed microtiter plates. After 48 hours incubation (37°C, 5% CO_2, 90% relative humidity) cells were pulsed with 0.5 μCi ^{3}H-thymidine (Amersham, spec. act. 25 Ci/mmol) 18 hours prior to termination of the cultures. Cells were harvested with an automated cell harvester and the amount of radioactivity incorporated into cellular DNA determined with the Filter Counting System (Inotech, Switzerland). All assays were done in triplicates and results are expressed as percent inhibition (after correction for spontaneous proliferation) compared to controls.

Mixed Lymphocyte Reaction (MLR)

Cells (2×10^5 in 100 μl culture medium) were cocultured with the same amount of mitomycin C treated RPMI cells in microtiter plates. The allogeneic response was assayed on day 4 of culture by ^{3}H-thymidine incorporation. Results are expressed as percent inhibition (after correction for spontaneous proliferation of the responder cells) compared to controls. All assays were done in triplicate.

Tumor Growth Inhibition (TGI)

In order to evaluate the suppressive effect of FK 506 on the autonomous proliferation, the murine Meth A sarcoma cell line was used. The cells were incubated at a cell concentraion of 1×10^5 cells/ml in culture medium in a final volume of 100 μl. After 48 hours of culture, cell proliferation was assayed by ^{3}H-thymidine incorporation for 4 hours. Results of triplicate cultures are expressed as percent inhibition compared to controls. FK 506 was a generous gift from Fujisawa Pharmaceutical Company Ltd. Stock solution (1 mg/ml DMSO) was diluted in RPMI 1640 to appropriate concentrations.

IgG Synthesis

Human peripheral leukocytes at a final cell concentration of 1×10^6/ml in RPMI culture medium (supplemented with 20% heat inactivated fetal calf serum, 41 mM glutamine, 2 mM L-arginine monohydrochloride, 1 mM oxaloacetate, 70 μM human insulin, 25 mM HEPES, 50 μM 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin) were placed into microtiter plates. The lymphocytes were stimulated with pokeweed mitogen (1%). After 7 days of culture (37°C, 5% CO_2, 90% relative humidity) termination was performed by centrifugation ($800\times g$, 10 minutes, room temperature). The supernatant was harvested and IgG was quantified by ELISA according to established methods. All assays were done in triplicate and results are expressed as percent inhibition compared to controls.

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