PAPULACANDINS, A NEW FAMILY OF ANTIBIOTICS WITH ANTIFUNGAL ACTIVITY

I. FERMENTATION, ISOLATION, CHEMICAL AND BIOLOGICAL CHARACTERIZATION OF PAPULACANDINS A, B, C, D AND E

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Papulacandin, a new antibiotic complex, active against *Candida albicans* and several other yeasts, was isolated from a strain of *Papularia sphaerosperma*. The fermentation, isolation, physico-chemical properties and biological activity of the five structurally related papulacandins A, B, C, D and E are reported. Papulacandin B, the main component, was assigned the formula of $C_{47}H_{64}O_{17}$.

In the course of our screening for new antibiotics we found that a strain of *Papularia sphaerosperma* (PERS.), HÖHNEL*[1], which belongs to the Deuteromycetes, produces a mixture of new antifungal antibiotics.[2] Five structurally related components, namely papulacandins A, B, C, D and E were isolated and purified by chromatographic techniques. The major components present were papulacandins A, B and C, which are strongly active against *Candida albicans* and several other yeasts, whereas papulacandins D and E are only minor components with lower activity. In this communication the fermentation, isolation, characterization and biological properties of the papulacandin complex are reported.

Fermentation

The antimicrobial activity of the culture broth was determined by the agar diffusion assay method with paper discs of 6-mm diameter. The test organism was *Candida albicans* K 1133.

For the best production of the papulacandins a well sporulated agar slant culture of *Papularia sphaerosperma* was used as a seed culture. The slant was washed with 5 ml of a 0.2 molar SÖRENSEN phosphate buffer pH 7.

The resulting mycelial-spore suspension was poured into a 500-ml Erlenmeyer flask with one baffle. It contained 100 ml of nutrient broth of the following composition: 2% soybean, 2% mannitol. The pH was adjusted before sterilization to 8.2 with 1 N NaOH. The flask was shaken at 250 r.p.m. and 23°C for 48 hours. For a second preculture, three 2-liter Erlenmeyer flasks with four baffles, each containing 500 ml of the soybean medium mentioned above, were inoculated with 25 ml each of the culture broth from the first preculture. The incubation was carried out at 120 r.p.m. and 23°C for 48 hours.

This second flask preculture (1.5 liters) served as an inoculum for the fermenter preculture. Conditions: 50-liter fermenter, containing 30 liters of the soybean medium, 750 r.p.m., 1 liter/liter/min. air throughput and 1 kp/cm^2 pressure, duration 48 hours at 23°C. The fermentation was carried out in a 500-liter fermenter with 4 baffles and a six-blade turbine. The conditions were: 450 r.p.m., 1 liter/liter/min air throughput, 1 kp/cm^2 pressure, 23°C. The nutrient broth, 300 liters of the soybean

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medium, was inoculated with 15 liters culture broth of the 48 hours old fermenter preculture.

The optimal production of the papulacandins occurred after about 60 hours, when the pH of the culture broth reached 6.7.

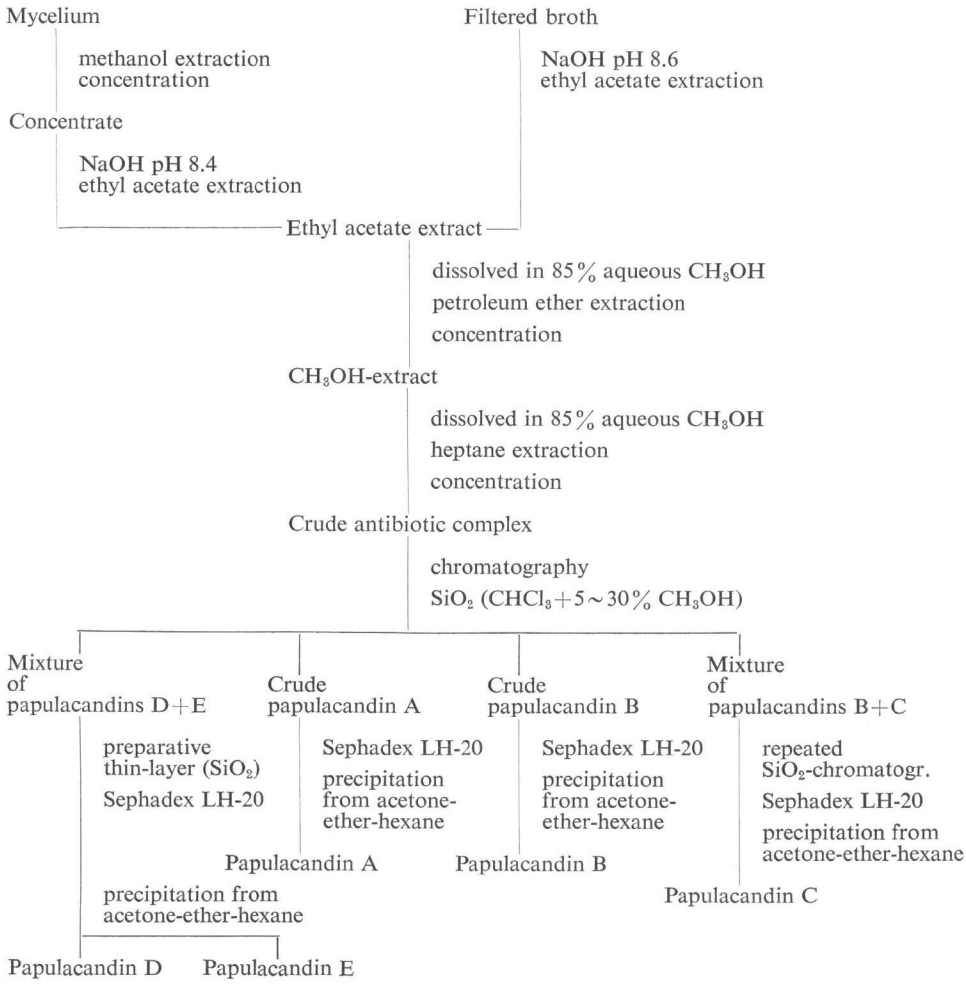
Isolation and Purification

The antibiotic complex was isolated from both the culture filtrate and the mycelium by extraction at pH 8.6 with ethyl acetate and methanol, respectively. The methanol extract from the mycelium was concentrated to an aqueous oil which was again extracted at pH 8.4 with ethyl acetate. Both ethyl acetate extracts were combined and concentrated to an oily residue.

The crude extract was dissolved in 85% aqueous methanol and lipophilic impurities were removed by extraction with petroleum ether and heptane. The methanol extract was concentrated again leaving the crude antibiotic mixture.

Further separation of the complex into its components was achieved by chromatography on silica-gel with chloroform and increasing amounts of methanol as eluents. Highly purified papulacandins A

Fig. 1. Isolation of papulacandin components



and B and mixtures of papulacandin B+C as well as papulacandin D+E were obtained in this manner.

Papulacandin C was separated from component B by repeated silica-gel chromatography of a mixture of the components B and C and pooling the fractions enriched in papulacandin C. This procedure afforded component C in about 90% purity.

The separation of the two minor components D and E was achieved by preparative silica-gel thin-layer chromatography ($CHCl_3$ - CH_3OH, 6: 1). Each component of papulacandin was finally purified by Sephadex LH–20 chromatography and precipitation from acetone-ether-hexane. A schematic representation of the isolation process is shown in Fig. 1.

General Characterization

All the components of papulacandin have very similar physico-chemical properties. They form amorphous colorless powders and are weakly acidic compounds. They dissolve well in lower alcohols, acetone, dimethylformamide and pyridine, are only slightly soluble in ethyl acetate, ethyl ether and chloroform and insoluble in benzene, petrolether, hexane and water. The Rf values of the five components of papulacandin in thin-layer chromatography are shown in Table 1. The physico-chemical properties of the papulacandins are summarized in Table 2.

Table 1. Rf values of papulacandins on thin-layer chromatography

Papulacandin	Solvent system	
	a	b
A	0.48	0.35
B	0.40	0.28
C	0.35	0.25
D	0.58	0.60
E	0.60	0.38

Solvent system a: Chloroform - methanol (4: 1) (repeated three times)
b: Ethyl acetate - acetone - water (72: 24: 4) (repeated twice)
Plates: Silica-gel F_{254}
Detection: UV, I_2, and bioautography with *Candida albicans*

The papulacandins are different in their physico-chemical properties from the polyenes[5] and the antibiotics echinocandin[3] and conocandin[4] recently isolated in our laboratories.

Physico-chemical Properties of Papulacandin B

Papulacandin B is the main component of the antibiotic complex. The UV spectrum shows three maxima at 232 (ε=42,000), 240 (ε=42,400), 268 (ε=44,800) nm and a shoulder at 300 nm (ε=

Table 2. Physico-chemical properties of papulacandins

Papulacandin	A	B	C	D	E
m.p.	171~173°C (dec.)	193~197°C (dec.)	140~150°C (dec.)	127~130°C	
$[\alpha]_D^{22}$ (CH_3OH)	+30±1°	+50±1°	+33±1°	+7±1°	not done
UV: $\lambda_{max}^{C_2H_5OH}$ (nm)	232 sh 242 265	232 240 268 300 sh	232 240 268 297 sh	230 235 261	230 sh 237 sh 267 292 sh
Elementary analysis C H O	62.29% 7.54% 29.40%	61.69% 7.20%	62.65% 7.16% 30.19%	62.32% 7.59%	
Molecular formula	$C_{47}H_{66}O_{16}$	$C_{47}H_{64}O_{17}$	$C_{47}H_{64}O_{17}$	$C_{31}H_{42}O_{10}$	unknown
Molecular weight	886	900	900	574	unknown

31,200). In the mass spectrum no molecular ion is visible. The infrared spectrum (Fig. 2) shows the presence of unsaturated carbonyl groups (1690, 1640, 1615 cm^{-1}) and hydroxyl groups (3500 cm^{-1}). In the 360 MHz–NMR-spectrum (Fig. 3) the signals of 3 methyl groups on saturated carbons at 0.9 ppm and one vinylic methyl group at 1.7 ppm are present. The signals in the region between 5.5 and 8 ppm

Fig. 2. Infrared absorption spectra of papulacandins A, B, C, D and E (KBr).

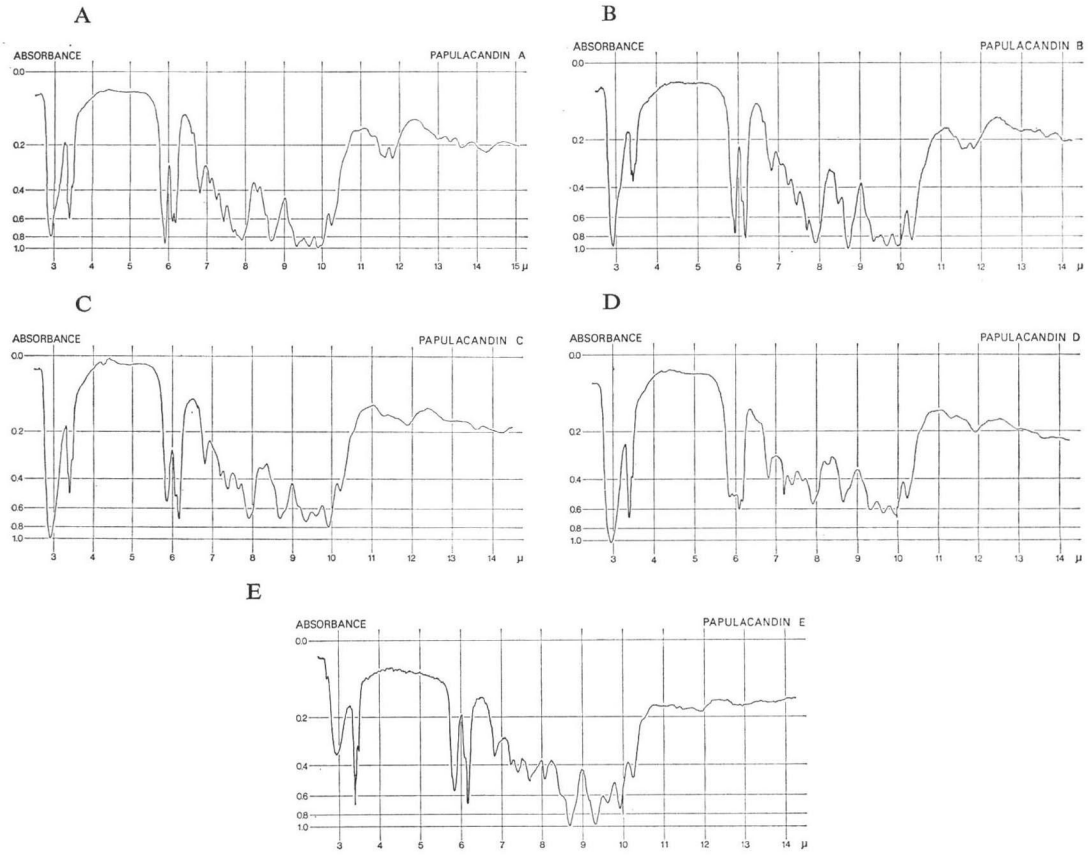
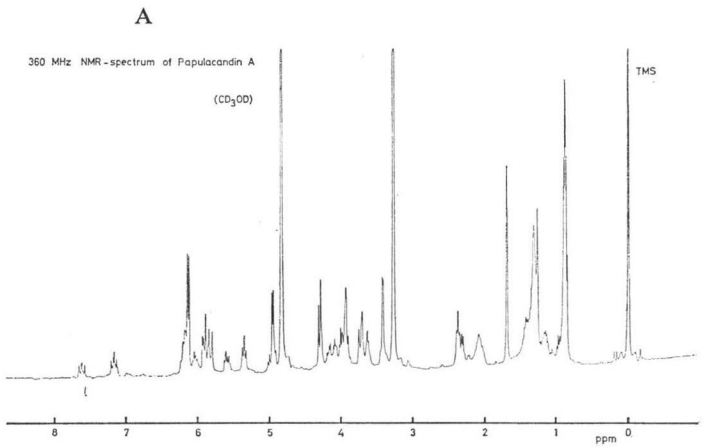
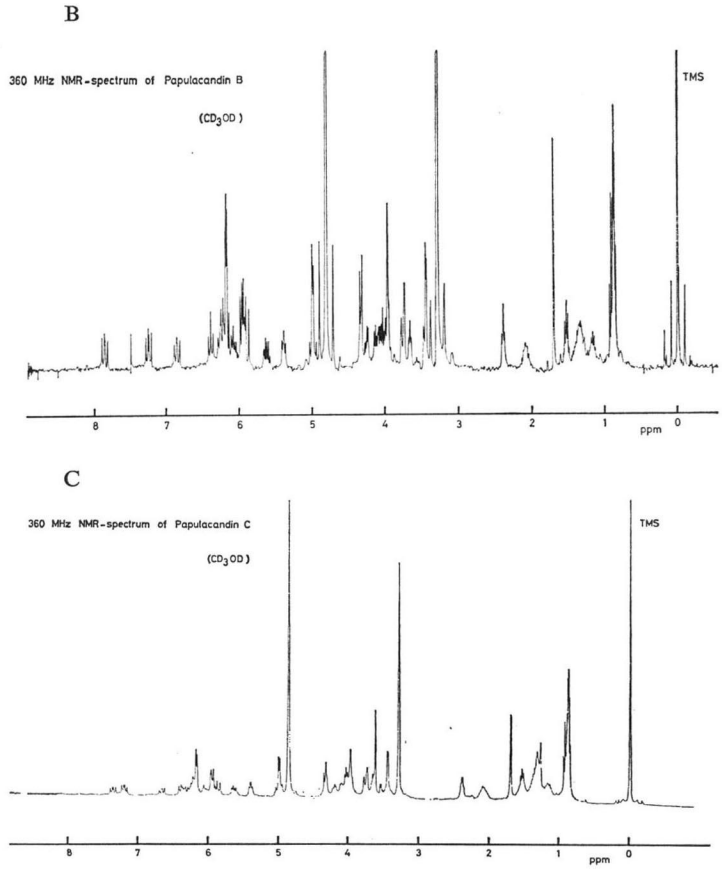


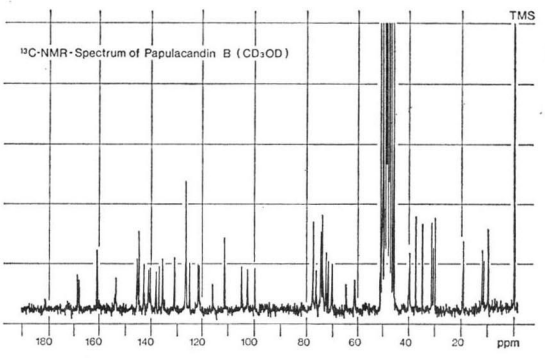
Fig. 3. 360-MHz-NMR Spectra of papulacandins A, B and C (CD_3OD).





can mostly be attributed to olefinic hydrogens. The two double doublets at 7.25 ppm (J=12/16 Hz) and 7.85 ppm (J=12/16 Hz) indicate olefinic hydrogens in β-position to a carbonyl group. In the ^{13}C–NMR-spectrum of papulacandin B (Fig. 4) the signals of 45~47 carbon atoms are found. The presence of several signals of methine and methylene groups α to oxygen in the ^{13}C- and ^{1}H-NMR-spectrum and the high number of alcoholic groups suggest the presence of at least one sugar moiety in the molecule. No free carboxyl and no O-methyl groups were found in the antibiotic.

Fig. 4. ^{13}C-NMR Spectrum of papulacandin B (CD_3OD).



The acetylation of papulacandin B with acetic anhydride in pyridine solution gave a fully acetylated nonaacetate. Nine acetate groups are visible in the 360 MHz–NMR-spectrum between 1.7 and 2.2 ppm.

By reaction with diazomethane papulacandin B gave both a monomethyl and a dimethyl derivative thus showing that two of the nine hydroxyl groups present in the molecule have an acidic character. The absence of any free carboxyl groups indicates that two phenolic hydrogens could be present.

Hydrogenation of the antibiotic gave an uptake of 7 moles of hydrogen. In the ^{1}H–NMR-spectrum of tetradecahydropapulacandin B all the olefinic hydrogens and the vinylic methyl group disappeared. The two signals at 6.2 ppm may be attributed to two aromatic protons in meta-position.

From elementary analysis and the ^{13}C-NMR spectrum of papulacandin B and its nonaacetate, and from preliminary degradation experiments, the molecular formula was determined to be $C_{47}H_{64}O_{17}$. Structural studies of papulacandin B by degradation experiments are in progress and will be reported elsewhere.

Biological Properties

The papulacandins have a high specific activity against yeasts. They are largely inactive against filamentous fungi and they show, with the exception of a slight activity against some gram-positive bacteria, no activity against bacteria and protozoa. The antimicrobial spectrum of the papulacandins is shown in Table 3.

The papulacandins A and C are about one half as active as papulacandin B, whereas the papulacandins D and E show a much lower antimicrobial activity.

Against *Candida albicans* papulacandin B exhibits a lower MIC than the well known antifungal substances amphotericin, nystatin and clotrimazol (see Table 3).

No cross resistance exists between the papulacandins and the polyenes[5] or the new antifungal antibiotic conocandin[4] or the chemotherapeutics clotrimazol[6] and miconazol[7]. A partial cross resistance, possibly based on a similar mode of action, has been seen between papulacandin B and echinocandin B, a new peptide antibiotic active against yeasts and fungi.[3,8]

There is evidence that the site of action of papulacandin B is the structural glucan of the yeast's cell wall.[2]

During our observations papulacandin B indicated a fungistatic action in the following test system: A proper MIC determination by a 1: 2 serial dilution test with *Candida albicans* in Mycophil broth was carried out. The contents of the last four tubes showing no visible growth, were filtered separately through 0.45 μ Millipore filter discs. After washing out the remaining antibiotic with Mycophil broth - 10 times of the original volume were used - the filter discs were incubated on Mycophil agar for 24 hours.

No growth on the discs was observed at concentrations higher than ten times the MIC level, while full growth was observed at lower levels. Therefore papulacandin was classified as a fungistatic antibiotic. On the other hand, by examining liquid cultures under the microscope, a fungicidal activity at MIC levels was also observed, though only on growing cells. The growing buds of these cells burst under the influence of papulacandin B, whereas resting cells remain unaffected.

In a soft agar diffusion system with a monolayer of chicken fibroblasts, a 5% solution of papulacandin B in dimethylformamide showed a very slight cytotoxicity. The fibroblasts in a 2-mm circle around a 6-mm Whatman filter paper disc, placed on top of the soft agar, were unable to accumulate Neutral Red, thus indicating a slight cytotoxicity of the substance.

The acute toxicity of papulacandins A and B in mice is very low. The LD_{50} (s.c.) is above 1,000 mg/kg. In *in vivo* tests a generalized infection of mice with *Candida albicans,* produced by an i.v. injection of 2×10^{6} viable cells/mouse, was cured by a subcutaneous application of papulacandin A or B. The ED_{50} was 180 and 80 mg/kg respectively. No curing effect was observed in oral treatments up to 1,000 mg/kg.

Table 3. Antimicrobial spectrum of the papulacandins and some other fungistatica (MIC values in mcg/ml, tested with the agar incorporation method)

Strain		Papulacandin				Clotrimazol	Amphotericin B	Nystatin
		A	B	C	D			
Candida albicans	K 335	0.4	0.1	0.4	6.2	0.8	0.8	3.1
Candida albicans	K 341	0.4	0.1	0.4	6.2	6.2	0.8	3.1
Candida albicans	K 1082	0.2	0.1	0.4	6.2	0.8	0.4	6.0
Candida albicans	K 1133	0.2	0.1	0.4	0.8	3.1	1.6	6.2
Candida guilliermondii	K 334	>100	>100	>100	>100	0.8	>100	>100
Candida tropicalis	K 337	0.4	0.1	0.8	1.6	12.5	1.6	3.1
Candida tropicalis	K 1155	0.8	0.2	0.8	12.5	6.2	1.6	3.1
Candida parapsilosis	K 332	0.8	0.1	0.8	1.6	0.2	1.6	3.1
Candida parapsilosis	K 1154	0.2	0.2	0.2	0.2	0.1	0.8	3.1
Candida utilis	K 482	0.4	0.2	0.8	1.6	0.8	0.8	1.6
Candida krusei	K 1153	0.8	0.2	0.8	12.5	6.2	3.1	3.1
Torulopsis dattila	K 336	>100	0.8	6.2	>100	6.2	1.6	0.8
Torulopsis famata	K 338	>100	0.2	1.6	>100	0.1	>100	25
Torulopsis glabrata	K 588	1.6	0.8	3.1	12.5	0.8	0.8	3.1
Saccharomyces cerevisiae	K 1085	1.6	0.4	3.1	12.5	0.4	1.6	1.6
Microsporum canis	K 240	0.8	0.8	1.6	12.5	0.8	1.6	6.2
Cryptococcus neoformans	K 340	>100	>100	>100	>100	0.2	0.1	1.6
Sporotrichum schenckii	K 83	>100	>100	>100	>100	3	10	6
Trichophyton mentagrophytes	K 84	>100	>100	>100	>100	0.3	10	6
Trichophyton rubrum	K 1087	>100	>100	>100	>100	12.5	3.1	6.2
Aspergillus fumigatus	K 76	>100	>100	>100	>100	0.8	10	12
Aspergillus niger	K 617	>100	>100	>100	>100	12.5	1.6	25

At 23°C the stability of a 0.1% solution of papulacandin B in Mycophil broth is highest between pH 5 and pH 7. At 4°C the solution is stable for more than 3 weeks over a much wider pH range: pH 3~pH 9.

Experimental

Derivatives

Acetylation: Papulacandin B (250 mg) was treated with 2 ml acetic anhydride and 2 ml pyridine for 3 hours at room temperature. The reaction mixture was then evaporated *in vacuum* and chromatographed on 30 g silica-gel with chloroform - methanol (99: 1) as solvent giving 200 mg of colorless, amorphous papulacandin B nonaacetate after precipitation from ether - hexane.
$[\alpha]_D^{22°} = +6 \pm 1°$ (*c* 0.865, $CHCl_3$); IR $\nu_{max}^{CH_2Cl_2}$: 1755 cm^{-1} (C=O, sat.), 1645 cm^{-1}, 1620 cm^{-1} (C=C), 1225 cm^{-1} (C–O–C); NMR (360 MHz, $CDCl_3$) : 9 visible acetate groups between 1.75 and 2.40 ppm; UV $\lambda_{max}^{C_2H_5OH}$ (ε) : 216 nm (23,200), 242 nm (25,600), 268 nm (27,600), 295 nm (shoulder).

Calculated for $C_{65}H_{82}O_{26}$ C 61.02%, H 6.46%
Found C 60.83%, H 6.49%

Hydrogenation: Papulacandin B (500 mg) was hydrogenated with 100 mg PtO_2 in 100 ml ethyl alcohol. After 4 hours at room temperature the uptake was 7.1 equivalents of hydrogen. The solution was filtered and evaporated to dryness. Chromatography on 100 g silica-gel with chloroform - methanol (9: 1) as solvent and precipitation from acetone - ether - hexane afforded 230 mg white amorphous tetradecahydropapulacandin B.

m.p.: 125~130°C; $[\alpha]_D^{22°} = +7 \pm 1°$ (c 0.214, CH_3OH); UV $\lambda_{max}^{C_2H_5OH}$ (ε) 270 nm (3100); IR ν_{max}^{KBr} 3500 cm^{-1} (OH), 1720 cm^{-1} (C=O, sat.); NMR (360 MHz, CD_3OD) δ ppm: 6.25 (2 H, aromatic), no olefinic protons, no vinylic methyl group.

Calculated for $C_{47}H_{78}O_{17}$	C 61.69%, H 8.60%, O 29.72%
Found	C 60.94%, H 8.56%, O 30.10%

Methylethers

Papulacandin B (2 g) was dissolved in 100 ml dioxane and treated with 100 ml of an 0.8 M diazomethane solution in ether for 30 minutes at 0°C. The mixture was evaporated to dryness and the residue chromatographed on 200 g silica-gel with chloroform and increasing amounts of methanol (5% to 20%). Fractions were combined according to their tlc giving 250 mg papulacandin B-dimethylether and 550 mg papulacandin B-monomethylether after precipitation from acetone-ether-hexane.

Papulacandin B-monomethylether: UV $\lambda_{max}^{C_2H_5OH}$ (ε): 235 nm (39,200), 265 nm (40,000), 302 nm (shoulder); IR ν_{max}^{KBr}: 3500, 1705, 1640, 1615 cm^{-1}; ^{1}H-NMR (CD_3OD) δ ppm: 6.29 and 6.34 (2 H, aromatic), 3.80 (s, 3 H, aromatic-OCH_3).

Papulacandin B-dimethylether: UV $\lambda_{max}^{C_2H_5OH}$ (ε): 230 nm (34,000), 265 nm (35,500), 305 nm (shoulder); IR ν_{max}^{KBr}: 3500, 1710, 1640, 1610 cm^{-1}; ^{1}H-NMR (CD_3OD) δ ppm: 6.48 (2 H, aromatic), 3.82 (s, 6 H, 2 aromatic-OCH_3).

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