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Pollitin is a high quality natural extract. extracted from rye pollen under the production and research with technology The same standard as the production of drugs according to the requirements of the World Health Organization. therefore has been registered as “NUTRACEUTICAL” or “nutritional therapeutic nutrition” receiving the ORAC standard or the antioxidant concentration and the CAP-e Test or the ability to be absorbed into red blood cells at a very high level

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Our premium natural extracts originate from meticulously selected flower pollen found in "Rye." These extracts undergo a unique proprietary production process crafted by Graminex L.L.C. in Ohio, United States. This exclusive process encompasses every stage, from cultivation and harvesting to the creation of high-quality natural extracts, specifically G60 and G63, derived from GBX flower pollen particles. Graminex holds the sole rights to this process and maintains adherence to strict pharmaceutical production standards in alignment with the World Health Organization's requirements.

Our extracts are renowned for their world-class production standards, boasting ORAC certification for exceptionally high antioxidant concentration and CAP-e Test accreditation, which signifies outstanding absorption into red blood cells. Over more than five decades, we have consistently refined and improved our product's efficacy.

Registered as a "NUTRACEUTICAL" or "nutritional therapy," Pollitin addresses issues at the cellular level, offering antibacterial properties and reinforcing immunity. By delivering essential nutrients tailored to various bodily systems, it equips the body to effectively combat abnormal cells. Our dedication to research is exemplified by over 150 certifications from medical and pharmaceutical institutions.

Moreover, Pollitin is not only a national achievement but a global triumph, available in over 50 countries. Our exclusive patented production process sets us apart as the sole producer of this unique formulation globally, rendering it impossible for anyone else to replicate our success in extracting and utilizing these flower pollen particles.

Pollitin - สารอาหารบำบัดเซลล์อ

สารสกัดธรรมชาติคุณภาพสูง สกัดจากเกสรดอกไม้จาก “ข้าวไรย์” ที่มีสูตรลับเฉพาะของ บริษัท (Graminex L.L.C.) ที่รัฐโอไฮโอ้ ประเทศสหรัฐอเมริกา ในการปลูก เก็บ และผลิตสกัดธรรมชาติคุณภาพสูง G60, G63 จากอณูละอองเกสรดอกไม้ GBX, Graminex® เอกสิทธิ์เฉพาะของบริษัท Graminex เท่านั่นที่ผลิตได้เพียงเจ้าเดียวในโลก อยู่ภายใต้การควบคุมมาตรฐานการผลิตยา ตามข้อกำหนดขององค์การอนามัยโลก

จนเราได้รับการรับรองมาตรฐานการผลิตระดับโลกระดับเดียวกับการผลิตยาเพราะ Pollitin ได้รับรองการทดสอบค่า ORAC หรือ ค่าระดับความเข้มข้นของสารต้านอนุมูลอิสระที่สูงมาก และ CAP-e Test หรือค่าความสามารถในการดูดซึมเข้าสู่เม็ดเลือดแแดงในระดับที่สูงจนได้รับ

การขึ้นทะเบียนเป็น “NUTRACEUTICAL” หรือ “โภชนเภสัช สารอาหารบำบัดระดับเซลล์” ที่สามารถแก้ไขปัญหาฟื้นฟูได้ลึกถึงระดับเซลล์ มีฤทธิ์ฆ่าเชื้อแบคทีเรีย และมีผลเสริมสร้างภูมิต้านทานเมื่อเซลล์ต่างๆ ได้รับสารอาหารที่เหมาะสมตามระบบต่างๆ ในร่างกาย ส่งผลให้ร่างกายสามารถต่อสู้กับ เซลล์ที่ผิดปกติภายในร่างกายได้ถึง 95% และยังได้รับรองมาตรฐานการผลิตและประสิทธิภาพจากองค์กรต่างๆ มากมายระดับโลก รวมไปถึงยังได้รับรางวัลการันตีอีกมากมายจาก เอกสิทธิ์สูตรลับพิเศษเฉพาะของ Graminex ทำให้สินค้ามีคุณภาพและเกิดผลลัพธ์ที่ดีและน่าเชื่อถือ จนได้รับการยอมรับระดับสากลอีกด้วย

ตลอดระยะเวลากว่า 50 ปี เราได้มีการวิจัยพัฒนาประสิทธิภาพอย่างต่อเนื่อง มีการวิจัยจากสถาบันทางการแพทย์และเภสัชกรรมรับรองมากกว่า 150 การวิจัย เรามีความภูมิใจอย่างมากในการเป็นผู้ผลิตหนึ่งเดียวของโลกที่ได้ครอบครอง ถือลิขสิทธิ์เอกสิทธิ์กระบวนการผลิตและสูตรเฉพาะ G60 และ G63 จากละอองเกสรดอกไม้ชนิด GBX ที่ไม่มีใครสามารถทำได้ ส่งผลให้ Pollitin เป็นที่ยอมรับจากคนจำนวนมากใน 6 ทวีป 50 ประเทศทั่วโลก และได้รับผลตอบรับที่ดีจากผู้บริโภคในการซื้อซ้ำสินค้าอย่างต่อเนื่องมากกว่า 50 ปี

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งานวิจัย เกสรดอกไม้ต่อโรคมะเร็ง

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Antitumour Potential of Pollen Extract on Lewis Lung Carcinoma Implanted Intraperitoneally in Syngeneic Mice

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A defined pollen extract of selected plants has been reported to possess some pharmacological activities on chronic prostatitis or benign prostatic hyperplasia. This paper describes the antitumour potential of the water-soluble fraction (Cernitin T60) of the pollen extract against Lewis lung carcinoma implanted intraperitoneally in syngeneic mice. Cernitin T60 was not cytotoxic in cell cultures at concentrations up to 2.5 mg/mL, while it is significantly prolonged the life-span of mice carrying the tumour without any apparent side effects at 0.5 g/kg. In addition, Cernitin T60 demonstrated beneficial therapeutic effects in an additive fashion on the life-span of mice when it was combined with standard cytotoxic antitumour drugs such as adriamycin, cisplatin, vincristine, methotrexate, fluorouracil, or thioguanine. The antitumour potential of Cernitin T60 was completely abolished by treatment with inhibitors of macrophage functions (2-chloroadenosine or carrageenan), but not with the T-cell inhibitor (cyclosporin A). Cernitin T60 appears to be a potent immunostimulator of macrophages.

Keywords: antitumour; pollen extract; murine long tumour; immunomodulator

INTRODUCTION

Pollen extract of selected plants has been widely used for nearly 30 years as a phytotherapeutic agent to chronic prostatis or bening prostatic hyperplasia (Ebeling, 1986; Buck *et al.,* 1989, 1990) based on its α-adrengic blockage (Kimura *et al.,* 1986) antiinflammatory properities (Ito *et al.,* 1984; Loschen and Ebeling, 1992) and inhibitory potential on hormone-dependent growth of tissues of benign prostatic hyperplasia in nude mice (Wagner *et al.,* 1992) or cultivated prostate cells *in virto* (Habib, 1992). Pollen extract also has been reported to reduce hepatotoxicity of paracetamol (Juzwiak *et al.,* 1992) and to prevent poisoning caused by organic solvents (Ceglecka *et al.,* 1992). Numerous substances have been isolated from pollen extract; amino acids, carbohydrates, deoxyribosides, enzymes, coenzymes, vitamins, minerals, trace elements, sterols (Kvanta, 1986; Nielsen *et al.,* 1957; Nielsen and Holmström, 1957) and polyunsaturated fatty acids (Seppänen *et al.,* 1989). Cernitin T60 contains water-soluble α-amino acids (6.0% - 9.2%) and other water-soluble substances of pollen extract.

We now report the antitumour potential of a pollen extract on a system of Lewis lung carcinoma implanted intraperitoneally (i.p) in syngeneic mice (Furusawa and Furusawa, 1985, 1989, 1990), and the beneficial effects in combination with standard chemotherapeutic drugs.

MATERIALS AND METHODS

KB cell cultures for cytotoxicity test. KB cell line, a human pharyngeal carcinoma, adopted as a standard cell line for screening cytotoxic agents in the National Cancer Institute (Goldin *et al.,* 1977) was used in tests. The procedure has been described previously (Furusawa and

Table 1. Cytotoxicity of Cernitin T60 on KB cell cultures: comparison with standard cytotoxic drugs

Cultivation period	Degree of cytotoxicity (0 to 4+) and dose/mL of culture medium									
	Cernitin T60				ADR	DDP	VCR	MTX	5-FU	DACT
	10 mg	5 mg	2.5 mg	1.25 mg	0.2 µg	2 µg	0.01 µg	0.05 µg	1 µg	0.002 µg
Day 1	2+	0	0	0	2+	2+	2+	2+	2+	2+
Day 2	3+	1+	0	0	3+	3+	3+	3+	3+	3+
Day 3	4+	1+	0	0	4+	4+	4+	4+	4+	4+
Day 4		2+	0	0						
Day 5		3+	0	0						
Day 6		3+	0	0						

Graded doses of Cernitin T60 or 2 Minimum Toxic Dose of cytotoxic drugs in 0.1 mL of MEM medium was added into the tube cultures (1 mL of culture medium) of human KB cells immediately after seeding of ca. 2 × 10 000 cells/mL, and incubated for 6 days. One seeding was done on day 3 (4 times dilution of cell numbers) in the presence of T60. Degree of cytotoxic effect (0 to 4+) was determined by the morphological criteria, density of cells, degrees of acid production (pH changes of medium) in comparison with the control cultures. ADR: Adriamycin, DDP: Cisplatin, VCR: Vincristine, MTX: Methotrexate, 5-FU: 5-Fluorouracil, DACT: Actinomycin-D. µg = 0.001 mg

Furusawa, 1985) and a brief description is included in Table 1. The culture medium was composed of Eagle's minimum essential medium (MEM) with 5% fetal calf serum and gentamycin.

Tumour-animal system for testing activities of agents. Lewis lung carcinoma was obtained from the National Cancer Institute and was maintained in syngeneic C57BL/6 mice in our laboratory. The tumour mass (2—3 g, 2—4 weeks old) was minced in 10 mL of MEM and forced through an 80-mesh screen with a 20-gauge needle. An aliquot (0.2 mL) of tumour homogenate containing 2—4 x 10^5 live tumour cells was injected intraperitoneally (i.p.) into young adult (18—20 g) C57BL/6 mice. They died of cancerous panperitonitis accompanied by disseminated solid tumour masses and massive ascitic bleeding within 10-20 days.

Preparation of agents. The water-soluble fraction (Cernitin T60) of defined pollen extract (product of SAPEC S.A., Barbengo, Switzerland) was dissolved in MEM culture medium for cytotoxicity test or in distilled water for i.p administration (0.1 mL/mouse). Adriamycin, cisplatin, vincristine, methotrexate, 5-fluorouracil, 6-thioguanine, 2-chloroadenosine, carrageen (Type III, kappa) and cyclosporine (cyclosporine A) were obtained from commercial sources. Drugs were freshly dissolved or suspended in distilled water before use or frozen until used.

Statistical analysis. The statistical significance of differences was analysed by Student's *t*-test for mean survival time (MST) and Fisher's exact test for ranking of survivors.

RESULTS

Cytotoxicity test of Cernitin T60 in KB cell cultures

To determine the degree of cytotoxicity, the graded doses of Cernitin T60, dissolved in MEM culture medium, were added to KB cell cultures. The results are shown in Table 1. It was found that Cernitin T60 was not cytotoxic at a high concentration of 2.5 mg/mL, although it became toxic at 5-10 mg/mL. Routinely, we designate a chemical or an extract of crude natural product origin as nontoxic when it was not cytotoxic at a concentration of 0.1-0.2 mg/mL, respectively. The nontoxicity of Cernitin T60 was also confirmed in murine cell line, BALB/3T3, NIH/3T3, and XC cells (data not listed)

Effect of Cernitin T60 on i.p. implanted Lewis lung carcinoma in syngeneic mice

Preliminary toxicity test of Cernitin T60 in normal mice demonstrated that a single i.p. injection of 80 mg (4—4.4 g/kg) was lethal and 5 multiple (once/every other day) injections of 30 mg dose (1.5—1.7 g/kg) were barely tolerable with marked loss of body weight, while the same multiple i.p. injections of 10 mg dose were apparently not toxic and did not affect the natural increase of body weight. We also administered the graded doses in the nontoxic range (10—0.04 mg/mouse), i.p., every-other-day schedule for a total of 5 injections, starting 1 day or 2 days after tumour implantation. The results are shown in Table 2. It was found that the therapeutic administration of Cernitin T60 significantly prolonged the survival time of mice at the broad range of dosage (0.2—10 mg), although total cure was not observed.

Effect of Cernitin T60 in combination with standard cytotoxic drugs on i.p. implanted Lewis lung carcinoma

The optimal dose of standard cytotoxic drugs for combination therapy in Lewis lung tumour system has already been established (Furusawa and Furusawa, 1990). Administration of cytotoxic drugs at the optimal single dose was done 1 or 2 days after tumour implantation, and treatment with Cernitin T60 at dose of 1.5.or 2.0 mg/mouse was started on day 1, 2 or 3 and continued every other day for a total of 4 or 5 injections. Table 3 shows the results. The combination therapy of Cernitin T60 with adriamycin (1μg: 1/100 of the maximum tolerable dose, MTD), cisplatin 10 μg: 1/15 MTD), vincristine (5μg: 1/4 MTD), 5-flurouracil (100μg: 1/10 MTD), methotrexate (200 μg: 1/2 MTD) or 6-thiogunine (150 μg: 1/2 MTD) significantly prolonged the survival time in an additive fashion, and some mice survived in a tumour-free state at the end of the observation period. These optimal doses of cytotoxic drugs, except methotrexate and 6-thioguanine, were relatively low and not produce any toxic signs even when combined with Cernitin T60. The optimal dose of methotrexate or 6-thioguanine was one-half of the MTD and produced some loss of body weight, but the combined regimen with Cernitin T60 did not show any further loss of body weight. This means that Cernitin T60 seems to be a safe supplemental agent in increasing the efficacy of chemotherapeutic drugs, without additional increase of drug toxicity in mice.

Effect of 2-chloroadenosine, carrageenan, or cyclosporine on the antitumour potential of Cernitin T60 on i.p. implanted Lewis lung carcinoma in syngeneic mice

As mentioned before, Cernitin T60 is noncytotoxic in cell cultures at a high concentration of 2.5 mg/mL (Table 1), while multiple administration at a low dose of 0.2 mg/mouse/day was effective against tumour cells *in vivo* (Table 2). Therefore, it is unlikely that the agent suppresses the tumour cell growth by direct contact, but instead indirectly by enhancing the host immune system, such as macrophage and/or lymphocyte lineages. The potential of Cernitin T60 with concomitant treatment with 2-choloadenosine or carrageenan, both specific inhibitors of macrophage functions (Schultz and Altom, 1986; Schultz *et al.,* 1986; Rumjunek and Brent, 1978; Neveu and Thierry, 1982), or with cyclosporine A, a specific inhibitor of T lymphocyte functions (DiPadova, 1989) was examined. The multiple administration of 2-chloroadenosine (0.1 mg, i.p.), carregeenan (0.25 mg, i.p.) or cyclosporine (2 mg, s.c.) was started on day 1 and followed by multiple administration of Cerniltin T60. Table 4 shows the results. The antitumour potential of Cernitin T60 was completely abolished by treatment with 2-chloroadenosine or carrageenan, but not with cyclosporine.

Table 2. Effect of Cernitin T60 on intraperitoneally implanted Lewis lung carcinoma in syngeneic mice: comparison with standard cytotoxic drugs

Agent	Dose/mouse and schedule, i.p.	MST ± SD days	Number of mice survived/total	ILS %
Control (experiment 1)		10.5 ± 2.2	0/10	
Cernitin T60	10 mg, days 1, 2, 4, 6, 8	18.3 ± 6.0[a]	0/10	74
Control (experiment 2)		11.5 ± 2.4	0/6	
Cernitin T60	5 mg, days 2, 4, 6, 8, 10	23.6 ± 8.6[a]	1/5	105
	1 mg, days 2, 4, 6, 8, 10	21.8 ± 6.7[a]	0/5	90
	0.2 mg, days 2, 4, 6, 8, 10	22.8 ± 7.9[a]	0/5	98
Control (experiment 3)		12.3 ± 2.8	0/12	
Cernitin T60	2 mg, days 1, 3, 5, 7, 9	23.9 ± 7.8[a]	0/12	94
	0.2 mg, days 1, 3, 5, 7, 9	16.3 ± 4.6[b]	0/6	33
	0.04 mg, days 1, 3, 5, 7, 9	14.2 ± 3.5	0/6	15
Control (experiment 4)		13.6 + 5.4	0/5	
Vincristine	10 μg (1/2 MTD), day 1	26.4 ± 9.2[a]	0/5	94
Control (experiment 5)		14.9 ± 4.4	0/10	
Cisplatin	10 μg (1/15 MTD), day 1	25.3 ± 14.1[b]	0/8	70
Control (experiment 6)		13.9 ± 2.9	0/9	
Adriamycin	2 μg (1/50 MTD), day 1	24.2 ± 11.6[b]	0/9	74
Control (experiment 7)		12.6 + 2.4	0/10	
5-Fluoroucracil	0.5 mg (1/2 MTD), day 1	20.4 ± 13.9[b]	0/5	62
Control (experiment 8)		13.2 + 2.9	0/5	
Methotrexate	0.5 mg (1 MTD), day 1	19.4 ± 6.0[b]	0/5	47

[a] $p < 0.01$, [b] $p < 0.05$ compared with controls. MST, mean survival time; ILS, increase in life span; MTD, maximum tolerable dose.
The $2–4 \times 10^5$ tumour cells were implanted intraperitoneally (i.p.) into syngeneic C57BL/6 mice (18-20 g body weight). Treatment with Cernitin T60 was started i.p. on the next day (day 1) or day 2 and continued until 8, 9, or 10, a total of 5 injections. Treatment with cytotoxic drugs was done i.p. on day 1 only. Observation was done until day 50.

Table 3. Effect of Cernitin T60 in combination with standard cytotoxic drugs on i.p. implanted Lewis lung carcinoma in syngeneic mice

Agent	Dose/mouse and schedule, i.p.	MST ± SD	Number of mice survived/total	ILS %
Control (experiment 1)		11.5 ± 1.6	0/10	
Vincristine 2 or 5 μg, day 1		16.9 ± 5.4[a]	0/10	47
Cernitin T60	1.5 mg, days 1, 3, 5, 7 or 2 mg, days 2, 4, 6, 8, 10	20.7 ± 6.0[a]	0/10	80
Vincristine + T60	same dose	32.3 ± 13.1[b]	3/10	181
Control (experiment 2)		12.1 ± 2.2	0/10	
Cisplatin 5 or 10 μg, day 1		19.7 ± 5.6[a]	0/10	63
Cernitin T60	2 mg, days 2, 4, 6, 8 or 2 mg, days 3, 5, 7, 9	19.6 ± 6.5[a]	0/10	62
Cisplatin + T60	same dose	34.4 ± 11.3[a]	2/10	184
Control (experiment 3)		11.5 ± 2.1	0/10	
Adriamycin 1 μg, day 1 or 2		15.9 ± 3.5[a]	0/10	38
Cernitin T60	2 mg, days 2, 4, 6, 8, 10 or 2 mg, days 3, 5, 7, 9, 11	18.1 ± 5.5[a]	0/10	57
Adriamycin + T60	same dose	37.6 ± 17.6[a]	4/10[b]	227
Control (experiment 4)		11.3 ± 2.0	0/10	
Fluorouracil 100 μg, day 1		21.9 ± 8.1[a]	0/10	94
Cernitin T60	2 mg, days 2, 4, 6, 8, 10	20.2 ± 6.9[a]	0/10	79
Fluorouracil + T60	same dose	37.0 ± 15.4[a]	3/10	228
Control (experiment 5)		11.3 ± 2.5	0/10	
Methotrexate 200 μg, day 1		19.2 ± 6.0[a]	0/10	70
Cernitin T60 2 mg, days 3, 5, 7, 9		17.9 ± 6.6[a]	0/10	58
Methotrexate + T60 same dose		44.2 ± 18.1[a]	4/10[b]	291
Thioguanine 150 μg, day 1		20.0 ± 5.0[a]	0/10	77
Thioguanine + T60	same dose	32.3 ± 16.4[b]	3/10	186

[a] $p < 0.01$, [b] $p < 0.05$ compared with controls vs. single agent only (T60 or drug), or single agent only vs. the combination (T60 + drug).
The $2–4 \times 10^5$ tumour cells were implanted intraperitoneally (i.p.) into syngeneic C57BL/6 mice (18-20 g body weight). The i.p. administration of cytotoxic drugs on day 1 or 2 was followed by i.p. administration of T60 on day 1, 2, or 3 and continued every other day for a total of 4 or 5 injections. Observation was done until day 50.
Experiment 1, 2, or 3 is sum of each two experiments. For example, experiment 1 is composed of two experiments which were done with different dose and schedule: 2 μg of vincristine, day 1 and 1.5 mg of T60, days 1, 3, 5, 7; 5 μg of vincristine, day 1 and 2 mg of T60, days 2, 4, 6, 8, 10; and 5 mice used in each group.

Table 4. Abrogation of antitumour activity of Cernitin T60 on i.p. implanted Lewis lung carcinoma in syngeneic mice by concomitant treatment with 2-chloroadenosine or carrageenan but not with cyclosporine

Agent	Dose/mouse and schedule, i.p.	MST ± SD	Number of mice survived/total	ILS %
Control (experiment 1)		10.1 ± 1.5	0/10	
Chloroadenosine 0.1 mg, i.p. days 1, 2, 3, 4, 6		11.5 ± 2.9	0/10	15
Cernitin T60	2 mg, i.p. days 2, 4, 6, 8	26.4 ± 16.0[a]	0/10	161
Chloroadeno. + T60	same dose	10.7 ± 1.5	0/10	6
Control (experiment 2)		11.2 ± 1.1	0/5	
Carrageenan 0.25 mg, i.p. days 1, 3, 5, 7		11.0 ± 0.7	0/5	−2
Cernitin T60 1.5 mg, i.p. days 1, 3, 5, 7		19.4 ± 7.3[a]	0/5	73
Carrageenan + T60	same dose	12.4 ± 3.2	0/5	11
Control (experiment 3)		13.7 ± 2.8	0/6	
Cyclosporine 2 mg, s.c., days 1, 2, 3, 4, 6		10.8 ± 0.5	0/5	−21
Cernitin T60 10 mg, i.p. days 1, 2, 3, 4, 6		30.0 ± 18.4	2/5	119
Cyclosporine + T60	same dose	31.4 ± 17.4[a]	2/5	129

[a] $p < 0.05$ compared with controls vs treated. s.c. subcutaneously.
The $2–4 \times 10^5$ tumour cells were implanted intraperitoneally (i.p.) into syngeneic mice (18–20 g). Each agent was administered i.p. or s.c. daily or every other day for a total of 4 or 5 injections. Chloroadenosine or carrageenan was preceded 1 h to T60 when administered on the same day.

DISCUSSION

The standardized pollen extract preparation of selected plants, recognized as a phytotherapeutic agent, has been shown to posses definite symptomatic efficacy on chronic prostatis or benign prostatic hyperplasia (Vahlensieck and Rutishauser, 1992). The pharmacological basis of action seems to be multiple, such as α-adrenergic blockage (Kimura *et al.,* 1986), antiinflammatory (Ito *et al.,* 1984; Loschen and Ebeling, 1986) and growth-inhibition on prostate cells (Habib, 1992) or tissues (Wager *et al.,* 1992). The pollen extract is also reported to reduce hepatotoxicty of drug (Juźwiak *et al.,* 1992) or poisoning of organic solvents (Ceglecka *et al.,* 1992).

We have demonstrated the antitumour potential of the water-soluble fraction (Cernitin T60) of pollen extract against Lewis lung carcinoma implanted i.p. in syngeneic mice. The system of Lewis lung carcinoma in syngeneic C57BL/6 semi-syngerenic BDF1 (C57BL/6 X DBA/2) mice was adopted for antitumour screening by the National Cancer Institute on 1975, and about 80% of the cytotoxic drugs that had been active on the Lewis lung tumour screen were reported to be active at a clinical level (Staquet *et al.,* 1983). In addition, we have found that Lewis lung carcinoma implanted i.p. in syngeneic mice is relatively sensitive to immunotherapy by exogenous immunostimulators of chemical or plant origin (Furusawa and Furusawa, 1989, 1990; Furusawa *et al.,* 1992). We have also found the Cernitin T60 possessed beneficial efficacy in an additive fashion on the survival time and the cure rate when combined with standard cytotoxic drugs. This suggests a possible clinical application of the pollen extract as an active supplemental agent in cancer chemotherapy without fear of additional toxicity. The optimal effective dose (2 mg/mouse = 100-111 mg/kg, every other day) which is 1/20 of the maximum tolerable dose (20 mg/mouse, daily) could be continuously administered without any side effects, even when combined with cytotoxic drugs. Cernitin T60 was not directly cytotoxic in cell cultures of human KB cells, murine BALB/3T3. NIH/3T3 or XC cells, but it seemed to kill tumour cells directly *in vivo* via activation of macrophage lineage of host immune system, which could be abrogated by the administration of 2-chloroadenosine or carrageenan, both specific inhibitors of macrophage function (Shultz *et al.,* 1986; Rumjanek and Brent, 1978). We did not observe a dependency of the antitumour action of Cernitin T60 upon T-cell activation which should have been abolished by

the concomitant administration of cyclosporine A, a potent immunosuppressor specific for T-cell lineage (diPadova, 1989). This is contrary to our previous finding that antitumor activity of polysaccharide of an edible mushroom origin was abolished by the pretreatment with cyclosporine in our Lewis lung tumour system (Furusawa *et al.,* 1992)

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REFERENCES

Buck, A. C., Cox, R., Rees, W. M., Ebeling, L, and John, A. (1990). Treatment of outflow tract obstruction due to benign prostatic hyperplasia with the pollen extract, Cernilton, a double-blind, placebo-controlled study. Br. J. Urol. **66,** 398-404.

Buck, A. C., Rees, W. M., and Ebeling, L. (1989). Treament of chronic prostatis and prostatodynia with pollen extract. Br. J. Urol. **64**, 496-499.

Ceglecka, M., Wójcicki, J., Gonet, B., Put, A., Kuzna-Grygiel, W., and Samochowiec, L. (1991). Effect of pollen extracts on prolonged posiioning of rats with organic solvents. Phytocher. Res. **5**, 245-249.

DiPadova, F. E. (1989). Pharmacology of cyclosporine (Sandimmune) V. Pharmacological effects on immune function: in vitro studies. Pharmacol. Rev. **41**, 373-405.

Ebeling, L. (1986). Therapeutic results of defined pollen extract in patients with chronic prostatis of BPH accompanied by chronic prostatis. In, Therapy of Prostatic, ed. By E. Schmiedt, J. E. Alken, H. W. Bauer, pp. 154-160.

Furusawa, E., Chou, S. C., Furusawa, S., Hirazumi, A., and Dang,Y. (1992). Antitumour activity of Ganorderma lucidum, an edible mushroom, on intraperitoneally implanted Lewis lung carcinoma in syngereic mice. Phytother. Res. **6**, 300-304.

Furusawa, E., and Furusawa, S. (1985). Antitumor activity of a natural product Viva-Natural, extracted from Undaria pinantifida on intraperitoneally implanted Lewis lung carcinoma. Oncology **42,** 364-369.

Furusawa, E., and Furuawa, S. (1989). Anticancer potential of Viva-Natural, a dietary seaweed extract, on Lewis lung carcinoma in comparision with chemical immunomodulators and on cyclosporine-accelerated AKR leukemia. Oncology **46**, 343-349.

Furusawa, E., and Furuawa, S. (1990). Antitumor potential of low-dose chemotherapy manifested in combination with immunotherapy of Viva-Natural, a dietary seaweed extract, on Lewis lung carcinoma. Cancer Lett. **50**, 71-78.

Goldin, A., Vendeitti, J. M., Carter, S. K. (1977). Protocols for in vivo screening systems and for in vitro KB cell culture screen. National Cancer Institute Monograph **45,** 147-150.

Habib, F. K. (1992). Regulation of prostate growth in culture with the pollen extract, Cernitin 60, and the impact of the drug on the EGF tissue profiles. In, Benign Prostate Disease, ed. By W. Vahlensieck and G. Rutishauser, p 115.-122. Thieme, New York.

Ito, R., Noguchi, K., and Yamashita, S. *et al*. (1984). Anti-inflammatory effects of Cernitin pollen-extract (Cernilton). Pharmacometrics (Jpn). **28,** 55-65.

Juzwiak, S., Rainska, T., Dutkiewica, T. B., *et al.,* (1992). Pollen extracts reduce the hepatotoxicty of paracetamol in mice. Phytother. Res. **6,** 141-145.

Kimura, M., Kimura, I., Nakase, K., Sonobe, T., and Mori, N. (1986). Micturition activity of pollen extract: contractile effects on bladder and inhibitory effects on urethral smooth muscle of mouse and pig. Planta Med. **2,** 148-151.

Kvanta E. (1968). Sterols in pollen. Acta Chem. Scand. **22,** 1261-1265.

Loschen, G., and Ebeling, L. (1992). Inhibition of the arachidonic acid metabolism by an extract from Rye pollen. In, Benign Prostate Diseases, ed. By W. Vahlensieck and G. Ruitshauser, pp. 65-72. Thieme, New York.

Neveu, P. J., and Thierry, D. (1982) Effects of carregeenan, a macrophage toxic agent, on antibody synthesis and on delayed hypersensitivity in the guinea pig. Int. J. Immunopharmacol. **4,** 175-179.

Nielsen, M., Grömmer, J., and Lunden, B. (1957). Investigations on the chemical composition of pollen from some plants. Acta chem. Scnad. **9**, 1672-1680.

Nielsen, M., and Holmström, E. (1957). On the occurrence of folic acid, folic acid conjugates and folic acid conjugases in pollen. Acta chem.. Scand. **11,** 101-104.

Rumjanek, V. M., and Brent, L. (1978). Immunosuppressive activity of carrageenan for cell-mediated responses in the mouse. Transplantation **26,** 113-118.

Seppänen, T., Laakso, J., Wójcicki, J., and Samochowiec, L. (1989). An analytical study on fatty acids in pollen extract. Phytother. Res. **3,** 115-116.

Schultz, R. M., and Altom, M. G. (1986). Macrophage involvement in the antitumor activity of a synthetic acyltripeptide (FK-565) against experimental lung carcinoma metastases. J. Immunopharmacol. **8,** 1986.

Schultz, R. M., Tang, J. C., Delong, D. C., Ades, E. W., and Altom, M. G. (1986). Inability of anti-asialo-GM1 and 2-choloradenosine to abrogate maleic anhydride-divinylether-induced resistance against experimental murine carcinoma metastases. Cancer Rers. **46,** 5624-5628.

Staquet, M. J., Byar, D, P., green, S. B., and Rozencweig, M. (1983). Clinical predicitivity of transplantable tumor systems in the selection of new drugs for solid tumors: rationale for a three-stage strategy. Cancer Treat. Rep. **67,** 753-765.

Vahlensiek, W., and Rutishauser, G. (ed.) 1992. Benign Prostate Diseases, pp. 1-207. Thieme, New York.

Wagner, B., Otto, H., Becker, S., Schröder, H., and Klosterhalfen, H. (1992). Experimental treatment studies with Cernilton N in human benign prostatic hyperplasia. In, Benign Prostatic Diseases, ed. By W. Vahlensieck and G. Ruthishauser, pp. 123-127. Thieme, New York.

Isolation and Characterization of a Cyclic Hydroxamic Acid from a Pollen Extract, Which Inhibits Cancerous Cell Growth in Vitro

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One fraction designated FV-7, in the water soluble ingredient if the pollen extract Cernilton was found to be inhibitory to the growth of a prostate cancer cell line. Characterization of FV-7 by high-resolution mass spectrometry and nuclear magnetic resonance identified as hydroxamic acid, 2,4-dihydroxy-2H-1, 4-benzoxazin-3 (4H)-one (DIBOA). To confirm this further, we synthesized an authentic sample of DIBOA and found subsequently that the synthetic DIBOA was structurally indistinguishable from FV-7. Furthermore, in a separate experiment we compared the in vitro effects of FV-7 and DIBOA on the growth of a prostate cancer cell line and found that in both cases the effect was inhibitory and that the inhibition curves obtained for both compounds were virtually identical.

Introduction

Cernilton is a multipotent extract from rye pollen which is commercialized by Cernitin SA, Lugano, Switzerland; it is one of the most popular phytotherapeutic drugs in Europe. The extract contains two main components, namely the hydrophobic Cernitin GBX1 fraction, both of which are devoid of allergens and other high molecular weight substances.

Clinical trials reported the efficacy of this drug in the treatment of benign prostatic hyperplasia (BPH) and chronic prostatitis[2] with no reported side effects. Subsequently, it was demonstrated in vitro that the water soluble Cernitin T-60 fraction was the active component in the "Cernilton" mixture as this inhibited the growth of prostate cancer cells and primary culture from BPH specimen.[3,4] In addition, in vivo studies on rats showed significant reductions in the weight of the rat prostate following an intake of Cernilton over a period of 21 days.[5] Significantly there was no change in any of the other organs of the animals tested.

More recently we have identified a number of constituent components in Cernitin T-60 of which only one fraction, designated V-7 (FV-7), was found to be biologically active with respect to its strong inhibitory effect on the growth of prostate cell lines;[6] this prompted us to identify and characterize this active ingredient. We now report on the isolation, characterization, and biological evaluation of one natural component present in the pollen extract and believed to be responsible for the biological activity of this drug.

Results

Purification of Biologically Active Fractions from a Pollen Extract (Cernitin T-60).

The aim of the present investigation was to single out one or more components present in a pollen extract (referred to as T-60 water soluble fraction) which could be responsible for the

growth inhibition of the human prostate cell line DU-145. The first step of purification consisted of the dialysis of the spissum through a spectrapor dialysis membrane with a cutoff limit of 1 kDa. Using the in vitro DU-145 cell growth inhibition assay, the material inside the bag proved to be inactive (as tested in the range of 0.1-0.5 mg/mL) and was discarded. All of the inhibitory activity was recovered in the diffusate.

Diffusate (2g) was then loaded onto a Sephadex G-25 superfine column. Up to eight fractions could be resolved. They were separately lyophilized, and the total recovery was ~90% of the starting material. Each fraction was assayed for the inhibitory activity: only one fraction (FV) was found to exhibit significant inhibitory activity toward DU-145 prostatic cancerous cell line at a concentration range of about 100 µg/mL.
Fraction V, which eluted at the same position as N-2-4-DNP-L-alanine (MW=255 Da) on the same column (i.e., 3.7- fold void volume), accounts for ~ 10% of the components present in the diffusate, i.e., ~3.6% (w/w) of the Cernitin T-60 fraction extract. Fractions IV and VI also exhibited some inhibitory activity, which was likely to be due to the cross-contamination by fraction V.

Purification of the Active Fraction V.

Pooled fraction V (~50mg from five to six preparative G-25 runs) was then loaded onto a Sephadex G-10 column, equilibrated, and developed in distilled water. Fraction V yielded eight subfractions. The inhibitory activity was found to be associated with the seventh subfraction only (elution volume = 9.2-fold void volume), at a concentration as low as 15-20 µg/mL. FV-7 accounts for ~ 13% of the whole FV, which in turn corresponds to .3% (w/w) of the whole Cernitin T- 60.

G-10 pooled fraction v-7 (500 µg) was further loaded on a Lchro CART C18 reverse phase column (4.6 mm x 100 mm) on a Varian 5000 liquid chromatograph to isolate the active compound to homogeneity, i.e., in a form suitable for chemical characterization. The eluting system was .1% TFA, pH 2.0, with an acetonitrile, was separated from several minor contaminants which were estimated to account for no more than 10% of the total fraction V-7, as monitored at 254 nm. The in vitro biological test confirmed that the major HPLC peak had dramatic inhibitory effect on DU-145 cells at 10 µg/mL.

Chemical characterization of the Natural Product Contained in Fraction V-7 Ultraviolet Spectrum (UV).

The UV spectra of the HPLC-purified component V-7 at pH 7.0 exhibited a shoulder at 279 nm, an absorption maximum at 253 nm, and a minimum at 232 nm.

Mass Spectrometry (MS).

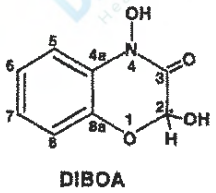
The strongest signals were obtained at 200° C during evaporation of the bulk of the sample. The spectrum showed a strong signal at *m/e* 181, which was thought to be the most reasonable candidate molecular ion (data not shown). High-resolution, narrow scan data provided an assigned mass of 181.0375. The only composition found within the maximum estimated error (0.1 mmu) was $C_8H_7NO_4$, 181.0375 calcd (error 0.1 mmu).

Nuclear Magnetic resonance (NMR).

The ^{1}HNMR spectrum of the natural product V-7 recorded in DMSO-d_6 showed seven ^{1}H resonances. Four of these, appearing as a multiplet at 7.0-7.3 ppm, immediately suggested the presence at 10.9 ppm was indicative of an acidic hydrogen atom. Two further resonances at 8.1 and 5.7 ppm, respectively, were found to couple with 5.5 Hz (vicinal coupling). Two hydrogen atoms of the molecule (at 10.9 and 8.1 ppm) underwent isotopic exchange with D_2O.

The ^{13}C-NMR spectrum showed eight resonances at 92.0, 112.9, 117.1, 122.5, 123.8, 128.7, 140.6, and 157.6 ppm. Together with the results from high resolution MS, these spectra definitely confirm the molecular formula $C_8H_7NO_4$ for the compound V-7. Two-dimensional heteronuclear shift correlation spectroscopy (H/C-COSY) revealed that only five of the eight C atoms are bound to hydrogen, i.e., three of the C atoms of the compound bear no H (those at 157.6, 140.6, and 128.7 ppm). The compound has neither a CH_2 nor a CH_3 group. Consequently, and in agreement with the isotope exchange experiment mentioned above, the two remaining H atoms must be bound to a heteroatom. The most reasonable fit between the structure and the NMR spectra was found for

2, 4-hydroxy-2H-1, 4-benzoxazin-3 (4H)-one (DIBOA).



DIBOA

DIBOA is a cyclic hydroxamic acid;[11] FV (G-25) and FV-7 (G-10) were the only two fractions of all the Sephadex fractions which exhibited a positive blue complex formation, characteristic for hydroxamic aids, with the ferric chloride spray method.[12] The ultimate proof that fraction V-7 was indeed identical to the known compound DIBOA resides in the independent chemical synthesis. This was carried out (see the Experimental Section), and the naturally occurring product V-7 was found indistinguishable by NMR, MS, UV spectra, and HPLC profile from an authentic synthetic sample of DIBOA.

Comparison of the Biological activity of V-7 and synthetic DIBOA.

At an initial cell density of 1.5 x 10^3 DU-145 cells/well, the inhibitory patterns for both the naturally occurring fraction V-7 and an authentic synthetic sample of DIBOA were tested at 1, 10, and 100 µg/mL. The results outlined in Figure 1 demonstrate an identical inhibitory pattern for the two compounds, thus confirming that there is no difference between DIBOA and V-7 in terms of their biological activities. At a concentration of 1 µg/mL of either V-7 of DIBOA, no growth inhibition can be observed from day 1-6. However at a concentration of 10 µg/mL of, the inhibitory effect at day 1 was found to be in the region of 50%, but this increased to 80% at day 5. When concentrations of 100 µg/mL inhibitory material were used, a complete shutdown of the proliferative effects was achieved from day 1 and this remained as such up to day 6. Similar patterns of inhibition were recorded when the initial cell density was increased to 2.5 x 10^3 cells/well (results not shown), suggesting that the inhibitory effect is independent of cell concentration.

Discussion

We described here the isolation of a cell growth inhibitor, designated fraction. V-7 from a commercial pollen extract, Cernitin T-60. V-7 was shown to be inhibitory at a concentration as low as 5-10 µg/mL when added to human prostatic cell line DU-145 in culture, and its content was evaluated to average 1% of the pollen extract.[6] High-resolution mass spectrometry and nuclear magnetic resonance allowed us to characterize V-7 as the cyclic hydroxamic acid, 2-4-dihydroxy-2H- 1, 4-benzoxazin- 3(4H)-one (DIBOA).[13,14] Furthermore, an authentic synthetic sample of DIBOA was found structurally indistinguishable from V-7. The in vitro comparative inhibition curves obtained with V-7 and DIBOA were virtually superimposable (Figure 1).

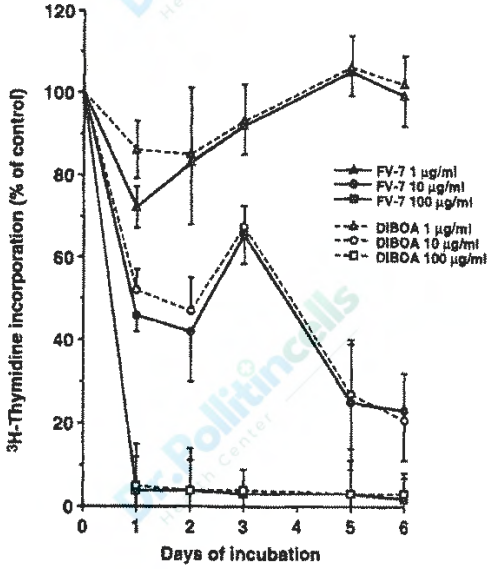


Figure 1. Growth of the androgen insensitive DU-145 human prostate cancer cell line following treatment with different concentrations of either fraction V-7 or DIBOA. Cells at a density of 1.5 × 10^3 cells/well were incubated for periods up to 6 days, and the results are expressed as percentage of thymidine incorporated relative to the untreated control. Each point is the mean of three separate experiments each run eight times. Bars represent coefficient of variation.

DIBOA is the enzyme hydrolysis product of the glycoside derivative.[13,14] It was recently shown that the parent glycosides of DIBOA and its methoxy derivatives occur in significant amount in some members of the Gramineae family of plants.[12] The amount of these compounds varies greatly depending upon species, age, and plant parts analyzed.[15] These cyclic hydroxamic acids

have however never been reported in pollen extracts from plants. Cernitin T-60 extract, which contains up to 95% pollen (w/w), appears to comprise at least one member of the DIBOA family. The cyclic hydroxamic acids have attracted much attention in agronomic research because of their role as phytotoxic agents,[16] even though the glycoside derivatives appeared to be less potent.

Until now, there were no reports in the literature concerning the role of DIBOA and its dihydroxy, methoxy, and glucoside derivatives as potential agents in the treatment of human neoplastic diseases. To the best of our knowledge, this is the first report on the use of DIBOA to inhibit human prostate cancer cell growth in culture. In this context, evaluation and antitumoral activities of hydroxamic acids have been reported. It was suggested that a series of polyhydroxy-substituted benzohydroxamates act as inhibitors of ribonucleotide reductase activity.[17-19] It remains to be seen whether the mechanism of action of DIBOA in the human prostate is similar. It is tempting to speculate that cell growth inhibition may result from the chelating or radical scavenger properties of DIBOA, which in turn may be the rate-limiting step of nucleotide biosynthesis; this is a possibility, which we are at present investigating.

Experimental Section

Cernitin T-60.

Cernitin T-60 is a pollen extract preparation from AB Cernelle, Helsingborg, Sweden. The water-soluble T-60 fraction accounted for more than 90% of the pollen extract.

Purification of Active Natural Product(s) from T-60 Fraction.

This was carried out by a combination of dialysis, gel filtration, and reverse phase HPLC steps, as outlined in ref.6. Dialysis of T-60 fraction was carried out against distilled water using spectra/Por (cutoff, 1 kDa) porous membranes. Contents inside and outside the bag were lyophilized. Twenty milliliters of the concentrated diffusate at a concentration of 200mg/mL was loaded onto a Sephadex G-25 superfine column (2.6 x 140cm) developed with distilled water at a flow rate of about 15mL/min, and the effluent was monitored at 280nm. The pooled biologically active material (20-30mg) was further fractionated through Sephadex G-10 column (2.0 X 100cm) using distilled water as eluent and monitoring at 280 nm. Biologically active subfraction from Sephadex G-10 chromatography was further purified to homogeneity by reverse phase HPLC on a semi-preparative Nucleosil C18RP column (1 x 25 cm); the eluting system was .01% aqueous TFA at pH 2.0 and an acetonitrile gradient was from 0 to 40% at a flow rate of 0.7mL/min. Monitoring was done at 254nm; 500-700 µg of the active subfraction from the G-10 step was repeatedly loaded, and the practical recovery was about 30-40%.

Physicochemical Characterization. Mass Spectrometry.

The mass spectrum was acquired by direct insertion on a MS 50S instrument (Kratos Ltd., Manchester, England) under conditions of electron impact ionization (70 eV) with a source temperature of 200° C.

Nuclear Magnetic Resonance.

The ^{1}H- and ^{13}C-NMR spectra of the active compound were recorded in deuterated dimethyl sulfoxide (DMSO-d_6) on a Bruker AMX-400 spectrometer (9.4T) operating at 400 and 100 MHz, respectively. Assignments were ascertained by two-dimensional homonuclear and heteronuclear shift correlation spectroscopy.[7]

Chemical Synthesis of the Active Compound.

An authentic sample of the active compound was obtained by an independent synthesis in four steps following procedures recently described.[8,9] The comparison of the physiochemical properties of the synthetic and the natural compound isolated from the T-60 fraction was made by HPLC, UV, MS, and NMR.

Biological Assay. Cell Culture.

To monitor and evaluate the inhibitory activity of each of the purified fractions from dialysis, gel filtration, and RP-HPLC steps, an assay employing the human prostate cancer cell line DU-145 was undertaken. Conditions for growth of these cells have been described previously as

well as thymidine incorporation and statistical analysis of the data.[6,10]

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References

1. Buck, C.; Cox, R.; Reece, R.W.M.,; Ebeling, L.; John, A. Treatment of outflow tract obstruction due to benign prostatic hypertrophy with the pollen extract, Cernilton: A double blind, placebo controlled study. *Br.J Urol.* **1990**, 66, 398-404.
2. Rugendoeff, E.W.; Weidner, W.; Ebeling, L.; Buck., A.C. Results of treatment with pollen extract (Cernilton) in chronic prostatitis and Prostatodynia. *Br. J. Urol* **1993**, 71, 433-438
3. Habib, F.K.; Ross, M.; Buck, A.C.; Ebeling, L.; Lewenstein, A. In vitro evaluation of the pollen extract, Cernitin T60 in the regulation of prostate cell growth,. Brit. J. Urol. **1990**, 66, 393-397.
4. Habib, F.K. Regulation of prostate growth in culture with the pollen extract, Cernitin T-60 and the impact of the drug on the EGF tissue profiles. In Benign Prostate Diseases; Ruthishauser, G., Vahlensieck, W., Eds.; George Thieme Verlag: Stuttgart, New York, **1992**; pp. 120-128.
5. Ito, R.; Ishii, M.; Yamashita, S.; Noguchi, K.; Kunikane, K.; Ohkuba, Y.; Tsushmima, K.; sato, S.; Akamatsu, H. Cernitin pollen extract (Cernilton), antiprostatic hypertrphic action of Cernilton pollen extract. *Pharmacometrics* **1986**, 31, 2-14.
6. Habib, F.K.; ross, M.; Lewenstein, A.; Zhang, X.; Jaton, J.C. The identification of a prostate inhibitory substance in a pollen extract. *Prostate* **1994**, in press.
7. Kessler, H.; gehrke, M.; Griesinger, C. Twodimensional NMR spectroscopy: background and overview of the experiments. *Angew*. Chem., Int. Ed. Engl. **1988**, 27, 490-536.
8. Sicker, D.; Pratorius, B.; Mann, g.; Meyer, L. A. convenient synthesis of 2, 4-Dihydroxy-1, 4-benzoxazin-3 (4H)-one. *Synthesis* **1989**, 211-212.
9. Atkinson, J.; Morand, P.; Arnaon, J. T.; Niemeyer, H.M.; Bravo, H. R. Analogues of the Cyclic Hydroxamic Acid 2, 4-Dihydroxy-7- methoxy- 2H-1, 4-benzoxazin-3-0one. *J. Org, Chem.* **1991**, 56, 1788-1794.
10. Macdinald, A.; Kabib, F.K. Divergent responses to epidermal growth factor in hormone sensitive and insensitive human prostate cancer cell lines. Br. J. Cancer **1992**, 65, 177-182.
11. Niemeyer, J.M. (Review). Hydroxamic acids (4-Hydroxy-1, 4-Benzoxazin-3-ones). Defence chemicals in the Gramineae. Phytochemistry **1988**, 27, 3349-3358.
12. Zuniga, G. E.; Copaja, S.V.; Bravo, H.R.; Argandona, V.H. Hydroxamic acid accumulation by wheat callus. Phytochemistry **1990**, 29, 2139-2141.
13. Virtanen, A.I.; Hietala P. K. Precursors of benzoxazolinone in rye plants, II. Precursor I, the glucoside. *Acta Chem. Scand.* **1960**, 14, 499-50.
14. Hietala, P.K.; Virtanen, A. I. Precursors of benzoxazolinone in rye plants, II. Precursor I, the glucoside. *Acra Chem. Scand.* **1960**, 14, 502-504.
15. Wolf, R.B.; Spencer, G.F.; Plattner, R.D. Benzoxazolinone, 2, 4-dihydroxy-1, 4-benzoxazin-3-one, and its glucoside from Acanthus Mollis seeds inhibit velvetleaf germination and growth. J. Natl. Prod. **1985**, 48 (1), 59-63.
16. Putnam, A.R.; Nair, M.G.; Barnes, J.P. Allelopathy: a viable weed control strategy. UCLA *Symp. Mol. Cell. Biol.* **1990**, 112, 317-322.
17. Elford, H.L.; Wamplet, G. L.; Riet, B. V. New nucleotide reductase inhibitors with antineoplastic activity. *Cancer Res.,* **1979**, 39, 844-851.
18. Elford, H. L.; Van't Riet, B. Inhibition of nucleoside diphosphate reductase by hydroxybenzohydroxamic acid derivatives. *Pharmacol. Ther.* **1985**, 29, 239-254.
19. Tihan, T.; Elford, H.L.; Gory, J. G. Studies in the mechanisms of inhibition of L1210 cell growth by 3, 4-dihydroxybenzohydroxamic acid and 3, 4-dihydroxybenzamidoxime. *Adv. Enzymes Regul.* 1991, 31, 71-83.

In Vitro Evaluation of the Pollen Extract, Cernitin T-60, in the Regulation of Prostate Cell Growth

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Summary— Nine human-derived cancer and non-cancer continuous cell lines were employed to evaluate the relative *in vitro* activity of the pollen extract, Cernitin T-60. Responses of the cell lines to the drug were assessed by measuring growth and cell survival as determined by cell count. The results demonstrated that of the 9 continuous cell lines tested, only those derived from the human prostate were growth inhibited by the pollen extract, whereas the non-prostate derived cells exhibited variable degrees of resistance to the T-60. The selectivity of the drug for the prostate cell lines was even more pronounced on the hormone- independent models, suggesting that there might be a place for the pollen extract in the control of abnormal growth in hormone-insensitive cells.

In spite of the considerable advances in our understanding of the processes leading to the growth and proliferation of the human prostate, the management of prostate diseases still remains a major clinical problem (Chisholm, 1989). Cancer of the prostate is the second most common cause of death due to cancer in males in the United Kingdom (Cancer Research Campaign, Factsheet 10.1, 1988) and the death rate is increasing. Clearly, the traditional forms of treatment such as surgery at the primary site, orchiectomy, hormone treatment and radiation are not as effective as Huggins might have originally perceived (Huggins and Hodges, 1941) and there is now every reason to find an alternative form of treatment.

Recently, there have been several reports suggesting that the pollen extract, Cernilton, is an effective agent in the treatment of prostate disease (Ito *et al*., 1986; Buck *et al*., 1989). The pollen extract is a preparation produced by AB Cernelle in Sweden and is essentially a microbial digestion of a mixture of pollens which have been extracted first in water and subsequently with an organic solvent (Kimura *et al*., 1986).

In an attempt to assess the selectivity and specificity of these pollen extracts, we undertook a number of experiments to compare the *in vitro* activity of Cernilton towards a wide range of human-derived cancerous and non-cancerous continuous cell lines of prostate and non-prostate origin. We confined our experiments to the water soluble fraction T-60 component, which accounts for approximately 60% of the pollen extract. In addition, we also undertook a few experiments on benign hyperplastic prostates to test the impact of the pollen extract on testosterone metabolism and the binding of androgens to their receptors.

Materials and Methods

Chemicals
Cernitin T-60 was a gift from AB Cernelle, Helsingborg, Sweden.

Tissues
Specimens of benign prostatic hyperplasia (BPH); obtained by transurethral resection, were transferred to the laboratory and either used immediately or snap frozen in liquid nitrogen and stored at -70° C.

Cell cultures
The epithelial and fibroblastic cell lines were all derived from human cancerous and non-cancerous tissue and details of their sources are

given in Table 1. Of the 3 human prostate cancer cell lines investigated, the LNCaP model is the only one which is hormonally responsive (Horosewicz *et al*., 1983), whereas the other 2 cell lines, the DU145 (Stone *et al*., 1978) and the 1013L (Williams, 1980) were all hormone-insensitive. All cell lines were maintained at 37°C under a humidified atmosphere at 5% CO_2 and 95% air in 75 cm^2 tissue culture flasks (Corning, New York, USA). The culture medium used was RPMI-1640 (Gibco, Paisley) supplemented with 10% (v/v) fetal calf serum, 20 mM HEPES, penicillin (100 units/ml), streptomycin (100 μg/ml) and 1% (v/v) L-glutamine. At each transplant, cells from the confluent monolayer were removed by trysinisation (trypsin 0.05%, EDTA 0.025%, Gibco) and suspended at 5 x 10^4 cells/ml in the growth medium.

Growth assays

Dose-response curves of Cernitin T-60 treatment were determined using the following method. Triplicate determinations for each treatment were performed in 24 well culture plates (Cell-Cult, Sterilin, Teddington). Each well was seeded with 5 x 10^4 cells and incubated overnight in the medium under incubation conditions as described above for routine cell culture. The following day, the T-60 stock solution was serially diluted in supplemented RPMI 1640 medium to yield concentrations of 1-4 mg/ml. Controlled cultures receive medium alone. For the dose-response curve studies, the cells were exposed to cernitin T-60 for a total period of 4 days, with changes of freshly diluted T-60 in medium every 2 days. For the time course study, cells were treated in the presence and absence of T-60 for 1, 2, 3, or 4 days. Experiments were terminated by the removal of cells from the monolayer by 2 successive trypsinisations and the pellets of harvested cells were subsequently suspended in 0.5 ml of Dulbecco A Medium (Oxoid Ltd, Basingstoke). The counting of cells was achieved on a haemocytometer slide after a 1-2 dilution with trypsin/ glutamine.

Nuclear androgen receptors

Methods used for the preparation of nuclear fractions and measurements of androgen receptors followed those previously published (Habib *et al*., 1986). For androgen receptor determinations, the competition binding assay was with 17α-methyl-^{3}H-methyltrienolone (R1881) in the presence of triamcinolone acetonide. Dissociation constants (Kd) and number of binding sites were determined by the Scatchard (1949) method.

Assay for 5α-reductase activity

5α-reductase was assayed at 37°C by following the conversion of (^{3}H) testosterone to (^{3}H) dihydrotestosterone and (^{3}H) 3α)β) androstanediol as previously detailed Habib *et al*., 1985).

Results

The effect of T-60 on cell growth

Proliferation curves of the hormone-insensitive and hormone-insensitive prostate cell lines in the absence and presence of increasing concentrations of T-60 for periods of up to 4 days are shown in Figure 1. Although the growth of each of these prostate cell lines was slowed following the addition of the pollen extract, the results show that the inhibition was much more

Table 1 Details of Cell Lines

Cell line	*Tumour type*	*Source*	*Cell/well*	*Duration of drug exposure (days)*
HEP	Cancer of the larynx	Gifts from Dr Mary Norval, University Medical School, Edinburgh	5×10^4	1–4
CHANG	Cancer of the liver		5×10^4	1–4
HEF	Human embryo fibroblast		5×10^4	1–4
RT112	Cancer of the bladder	Dr J. R. W. Masters, Department of Pathology, St Paul's Hospital, London	5×10^4	1–4
SUZA	Cancer of the testis		5×10^4	1–4
DU145	Cancer of the prostate	Gifts from Dr D. Mickey, Department of Urologic Research, University of North Carolina, USA	5×10^4	1–4
1013L	Cancer of the prostate		5×10^4	1–4
LNCaP	Cancer of the prostate	Gift from Dr J. S. Horoszewicz, Department of Medical Virology and Oncology, Roswell Park Memorial Hospital, Buffalo, USA	5×10^4	1–4
MCF-7	Cancer of the breast	Gift from Dr W. R. Miller, Department of Clinical Surgery, University Medical School, Edinburgh	5×10^4	1–4

marked in the case of the androgen-insensitive cell lines. Indeed, at 1 mg/ml the pollen had no effect on the growth of the LNCaP cells, which exhibited an identical profile to that of the control, whereas the androgen-insensitive 1013L and DU145 cells demonstrated significant inhibition, particularly on day 4. By contrast, at the higher pollen concentrations (4 mg/ml) the growth of all 3 prostate cell lines was arrested and the cell numbers were rapidly depleted with the time of exposure. After 4 days, cell counts had been reduced by an average of 94% compared with controls.

Parallel experiments on the non-prostate derived cell lines showed no response to pollen extract (1 mg/ml) even after 4 days' exposure (Fig.2). However, at the higher concentrations (4 mg/ml) the pollen induced some inhibition with the HEF and RT112 cells ($P<0.01$) following a 4-day incubation (Fig. 2), although this was not as marked as in the prostate cells. Significantly, none of the other non-prostate derived cells showed any significant response ($P>0.5$).

The effect of T-60 on androgen metabolism and steroid receptors

We also tested the impact of increasing concentrations of cernitin T-60 (0-10 mg/ml) on the 5α-reductase activity of tissue obtained from 6 separate BPH patients. As demonstrated in Table 2, there was no change in the activity of the enzyme with increase in T-60 even at concentrations as high a 10 mg/ml.

Table 2 Effect of T-60 Concentration on 5α-Reductase Activity of the Human Benign Prostate

Patient no.	*T-60 concentration (mg/ml)*			
	0	*0.75*	*2*	*10*
1	1.44 ± 0.2*	1.34 ± 0.23	1.38 ± 0.12	1.25 ± 0.09
2	1.55 ± 0.18	2.08 ± 1.10	0.98 ± 0.12	1.58 ± 0.29
3	6.29	6.98 ± 2.72	8.46 ± 1.29	9.89 ± 0.89
4	2.21 ± 0.15	2.18 ± 0.19	—	2.23 ± 0.23
5	2.98 ± 0.52	3.18 ± 0.21	4.45 ± 0.56	—
6	2.58 ± 0.26	2.4 ± 0.24	2.32 ± 0.04	2.28 ± 0.65

* Values expressed in pmol/mg protein/min ± SD.

Table 3 Effect of Cernitin T-60 (4 mg/ml) on Nuclear Androgen Receptor Measurements in 6 BPH Specimens

Treatment	*K_d (nmol/l ± SD)*	*Binding site (fmol/g tissue ± SD)*
Control	2.95 ± 0.60	84.4 ± 27.5
T-60 added	2.80 ± 0.57	78.8 ± 32.1

In addition, we undertook several experiments to measure nuclear androgen receptor levels in the absence and presence of the pollen extract at 4 mg/ml. The results summarised in Table 3 indicate that there was no significant difference between the control and test groups with regard to the number of binding sites ($P> 0.5$) and dissociation constants ($p>0.5$).

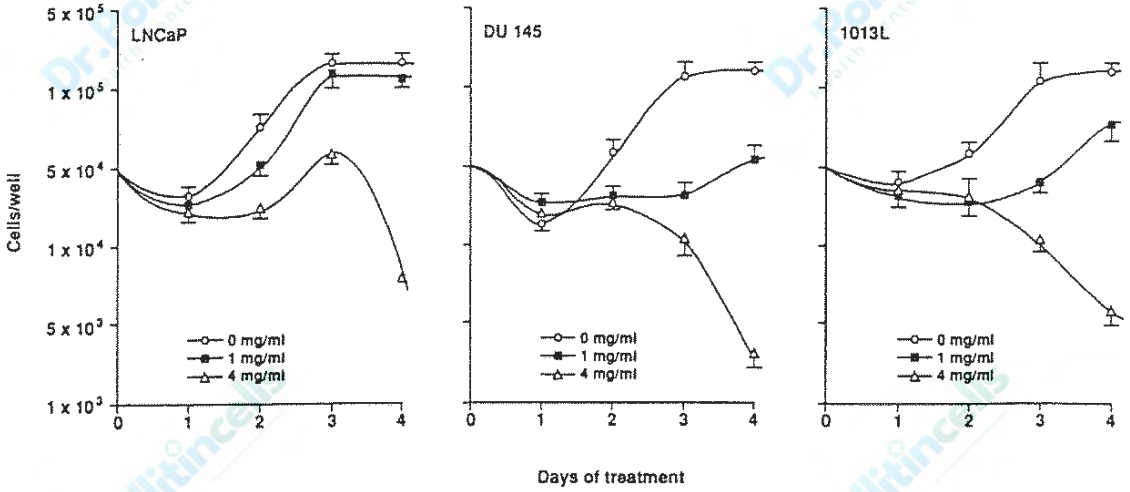

Fig. 1 The effects of varying the concentrations of Cernitin T-60 on the growth of androgen-sensitive and androgen-insensitive prostate cell lines. Each point represents the mean ± SD of 3 separate experiments each run 6 times.

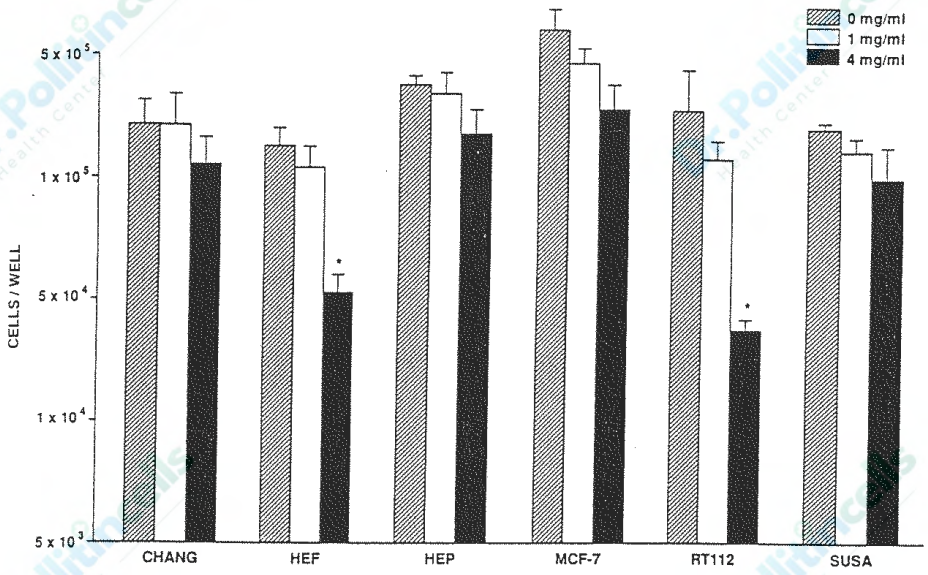

Fig. 2 The effect of Cernitin T-60 on the growth of 6 non-prostate derived cell lines after 4 days' exposure to the drug. Results are the mean ± SD of 3 separate experiments each run 6 times ($P < 0.01$).

Discussion

These data represent the first report of the *in vitro* evaluation of the water-soluble fraction of the pollen extract, Cernitin T-60, using a panel of human prostate tumour-derived continuous cell lines. In addition, parallel *in vitro* experiments were also undertaken on 6 other cell lines derived from non-prostatic sources essentially to assess the specificity and efficacy of pollen extract.

Attempts to minimise variations between experiments were made by standardising experimental conditions with regard to the same medium, fetal calf serum concentration, and narrow range of cell passages. Furthermore, we observed a little variation in drug response with repeated experiments for each particular cell line. Nonetheless, the results of this study suggest that the responses induced were varied and these were predominantly a function of the cell lines: high in the case of the prostate, low or non-existent in the non-prostate derived cells. Of interest also is the heterogeneity in responses of the prostate cell lines to the agent. The hormone-insensitive cells demonstrated a greater sensitivity to the pollen extract than the androgen-dependent line and this was particularly evident at the lower pollen concentrations.

We are not yet sure of the mechanism of action of this drug but quite obviously it is not mediated via the androgen delivery system of the cell, since the pollen had no effect on either the 5α-reductase activity of the tissues or its steroid receptors. There have also been reports suggesting that Cernilton might be a potent inhibitor of the cyclo-oxygenase and lipoxygenase enzymes which are needed for leucotriene and prostaglandin synthesis (Loschen, personal communication) but these reports have not been extended to the prostate and will require verification.

However, it is gratifying to note that the selectivity of the pollen extract for the prostate, as demonstrated in the present study, was also supported by the work carried out by Ito *et al*. (1986). Following an intake of Cernilton over a period of 21 days, the rats in the latter study showed significant reductions in the weight of the ventral and dorsal prostate but there was no change in any of the other major organs. Following these encouraging results, a double-blind trial was undertaken on a group of patients with BPH, the results of which are described by Buck *et al.* (1990).

References

1. Buck, A.C. Cox, R., Rees, R. W. M. *et al*. (1990). Treatment of outflow tract obstruction due to benign prostatic hypertrophy with the pollen extract, Cernilton. A doubleblind, placebocontrolled study. Br. J. Urol., 66, 398-404.
2. Buck, A.C., Rees R.W. M. and Ebeling, L. (1989). Treatment of chronic prostatitis and prostatodynia with pollen extract. Br. J. Urol., 64, 496-499.

3. Chisholm, G.D. (1989). Benign prostatic hyperplasia. The best treatment. Br. Med. J., 299, 215-216.
4. Habib, F.K., Odoma, S., Busuttil, A. *et al.* (1985). 5α-reductase activity in human prostate cancer is related to the histological differentiation of the tumour. *Clin. Endocrinol.*, 23, 431-438.
5. Habib, F.K., Odoma, S., Busuttil, A. *et al.* (1986). Androgen receptors in cancer of the prostate, correlation with the stage and grade of the tumor. *Cancer*, 57, 2351-2356.
Horoszewicz, J. S., Leong, S. S., Kawinski, E. *et al.* (1983). LNCaP model of human prostatic carcinoma. *Cancer Res*., 43, 1809-1818.
6. Huggins, C. and Hodges, C.V. (1941). Studies on prostatic cancer. I. The effect of castration, of estrogen, and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *Cancer Res*., 1, 293-297.
7. Ito, R., Ishi, M., Yamashita, S. *et al*. (1986). Cernitin pollen extract (cernilton): antiprostatic, hypertrophic action of cernitin pollen extract. *Pharmacometrics*, 31, 2-14.
8. Kimura, M. Kimura, I., Nakese, K. *et al*. (1986). Micturition activity of pollen extract: contractile effects on bladder and inhibitory effects on urethral smooth muscle of mouse and pig. *Planta Medica*, 2, 148-151.
9. Scatchard, G. (1949). The attractions of proteins for small molecules and ions. *Ann* N.Y. *Acad*. *Sci*., 51, 660-672.
10. Stone, K.R., Mickey, D.D., Wunderli, H. *et al.* (1978). Isolation of a human prostate carcinoma cell line (DU145). *Int. J. Cancer*, 21, 274-281.
11. Williams, R.D. (1980). Human urologic cancer cell lines. *Invest. Urol*., 17, 359-363.

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The Secalosides, Novel Tumor Cell Growth Inhibitory Glycosides from a Pollen Extract

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The pollen of rye (Secale cereale) was shown to contain a biologically highly active family of glycosides called the secalosides. Secalosides A and B (**1**), both of molecular formula $C_{46}H_{51}NO_{24}$, were found to be epimeric esters of (2-oxo-3-indolyl)acetic acid (**4**). They are made up, in addition to this heterocyclic aglycon I (**4**), of three hexose building blocks and a carbocyclic aglycon II, which is an indan-derived dicarboxylic acid (**5**). In aqueous solution, secalosides A and B interchanged by epimerization at the chiral center of **4**. A further epimeric pair, secalosides C and D (**2**), contain one additional glucose building block. Secalosides A and B, the racemic aglycon I (**4**), and 2-oxo-1,2,3,4-tetrahydroquinoline-4-carboxylic acid (**3**), which results from **4** by hydrolytic rearrangement, exhibited significant antitumor activity against S180 sarcoma *in vivo.* IC_{50} values obtained were about 5 μg/mouse for the secalosides and 1 μg/mouse for **3** and **4**.

Cernilton is an extract from rye pollen that is produced commercially and used as a phytotherapeutic drug in Europe. This highly heterogeneous extract comprises two main components, namely, the hydrophilic Cernitin T-60 fraction and the hydrophobic Cernitin GBX1 fraction, both of which are devoid of allergens and high molecular weight substances.

Clinical trials have shown the efficacy of Cernilton in the treatment of benign prostatic hyperplasia[1] and chronic prostatitis[2] without reported side effects. Cernitin T-60 was found to be the active fraction *in vivo,* as it inhibited the growth of prostate cancer cells.[3,4] Recent reports have described the isolation and characterization of a prostate growth inhibitory substance in the Cernitin T-60 fraction, namely, the cyclic hydroxamic acid, 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA).[5,6]

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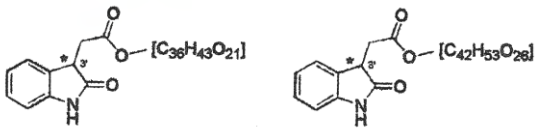
In another series of experiments carried out *in vivo*, the antitumor potential of Cernitin T-60 was demonstrated on Lewis lung carcinoma implanted extraperitoneally in syngeneic mice.[7] Similar results were obtained with mice implanted with S180 sarcoma. The survival time of mice treated with Cernitin T-60 exceeded 100% with the percentage of cured mice being 20-40%. However, synthetic DIBOA, although highly cytotoxic against a large variety of human tumor cell lines in culture, was found not to be inhibitory in *in vivo* Lewis lung carcinoma or S180 sarcoma implanted in mice (Sirotnak, F. M. Unpublished data).

These encouraging data prompted us to identify the ingredients present in Cernitin T-60 that are active *in vivo*. We now report on the isolation and biological evaluation of a family of glycosides shown to be responsible for the *in vivo* biological activity of Cernitin T-60. We propose that these compounds be called secalosides A-D by virtue of their origin from rye pollen (*Secale cereale* L., Gramineae). The structures of the constituent aglycons as well as a partial gross structure for intact secalosides are presented herein. It is

considered that one of these aglycons is responsible for most of the biological efficacy of the intact glycosides. The complete structure elucidation of secalosides A and B is the subject of the following paper in this issue by Juan *et al*.[8]



Secalosides A/B
1

Secalosides C/D
2

Results and Discussion

The strategy for the isolation of active compounds present in the Cernitin T-60 extract is summarized in Figure 1. A functional *in vivo* assay was used to monitor the biological activity through the various fractionation steps (see the Experimental Section). The compounds from peaks 4.51 (secaloside A) and 4.52 (secaloside B) were found to be equally active *in vivo*. NMR spectroscopy in water and HPLC unveiled a slow interconversion of the two compounds suggesting equilibrium between isomers. Similarly, another pair of isomers (peaks 3.51 and 3.52), which were called secalosides C and D, was isolated from the initial fraction 3 of the gel filtration. This biologically active pair was found to be structurally related to the secalosides A and B (*vide infra*).

FAB-MS gave an identical molecular weight of 1001 Da for secalosides A and B, while that of both the secalosides C and D was 1163 Da. The UV spectra of both compound pairs, recorded in water, were virtually indistinguishable with maxima at 280 nm and minima at 265 nm. Chemical microdeterminations[9,10] suggested that none of the secalosides contained an α-amino acid, a hydroxamic acid, or a fatty acid as a constituent. They all stained positively for phenolic compounds and for carbohydrates.[9,10] Acid hydrolysis in 1 N HCl for 6 h at 100 °C qualitatively unveiled the presence of $_{D}$-glucose in both pairs of secalosides, as determined by a specific enzymatic assay. In the enzymatic tests, none of the various α- and β-glycosidases (see the Experimental Section) hydrolyzed hexoses from secalosides A or B.

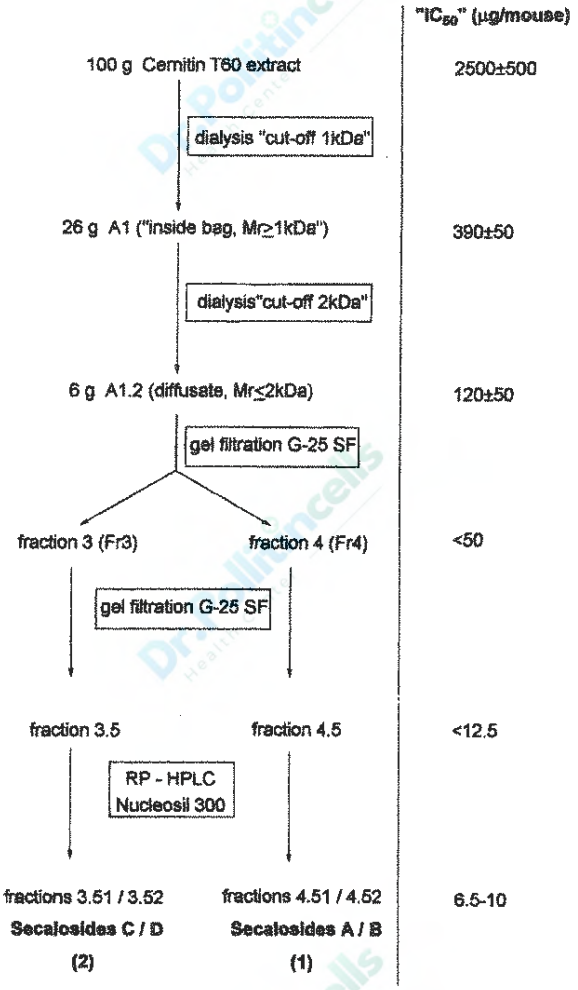


Figure 1. Fractionation steps employed to isolate the secalosides A–D from Cernitin T-60. "IC_{50}" values represent the inhibitory concentration of various fractions leading to a 50% reduction of S180 tumor cell growth as compared to untreated mice.

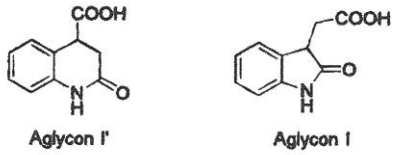
In contrast, when the mixture of secalosides C and D was digested for 16 h at 37 °C in the presence of α-glucosidase in phosphate buffer at pH 6.8, $_{D}$-glucose was released. Quantitative measurements indicated that 0.85 and 1.05 mol of glucose were released per mole of secaloside C and D, respectively. The digest was further analyzed by RP-HPLC, which indicated that the α-glucosidase treatment yielded a new pair of compounds eluting with retention times indistinguishable from those of the intact secalosides A and B. This result is consistent with the difference of 162 mass units between the molecular weight of secalosides A and B and secalosides C and D.

The products of the acid hydrolysis mentioned above (1 N HCl, 6 h at 100 °C) were analyzed by RP-HPLC and monitored at 250 nm.

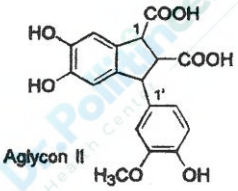
Hydrolysates of both pairs of secalosides (A and B and C and D) gave rise to the same elution profile, demonstrating the presence of two aglycons. Aglycon I (**4**) with a UV absorption maximum in water at 250 nm exhibited from its HREIMS the molecular formula $C_{10}H_9NO_3$. We consider that the genuine aglycon I underwent, under the hydrolysis conditions, an isomerization into aglycon I' (**3**). For this reason, the HPLC profile showed two peaks for this aglycon in a time-dependent ratio (*vide infra*). Aglycon II (**5**) had a UV absorption maximum in water at 282 nm and a minimum at 256 nm. Its HREIMS led to the apparent molecular formula $C_{18}H_{14}O_7$. Later on it was recognized that aglycon II lost one molecule of water in the spectrometer inlet prior to ionization, so that its definitive formula therefore is $C_{18}H_{16}O_8$.

These findings suggest that the isomeric secalosides A and B are made of five building blocks, namely one molecule each of aglycon I and aglycon II and three hexose subunits. This is consistent with a molecular formula of $C_{46}H_{51}NO_{24}$, which is in full agreement with the experimental molecular mass of 1001 Da.
Secalosides C and D, in addition, contain one extra glucose subunit; they have the molecular formula $C_{52}H_{61}NO_{29}$, in agreement with the experimentally determined molecular mass of 1163 Da.

An analytical sample (1.2 mg) of the nitrogen containing aglycon I' was isolated by preparative HPLC from the products obtained by acid hydrolysis of secalosides A and B. The compound was identified by standard spectroscopic means as racemic 2-oxo- 1,2,3,4-tetrahydroquinoline-4-carboxylic acid (**3**). The assignment was confirmed by an independent synthesis following a literature procedure.[11,12]
Nevertheless, various observations suggested that the structure of **3** did not properly reflect the constitution of the corresponding building block within the native glycosides. The HPLC profile of the hydrolysis products had shown next to **3** the peak of a transient compound with a similar retention time. Moreover, the NMR spectra of the glycosides (*vide infra*) are at variance with what one might expect, were the skeleton of **3** to be present. We therefore considered compound **3** to be an artifact resulting from a rearrangement during the hydrolysis procedure. Its most likely precursor is (2-oxo-3-indolyl)- acetic acid (OXIAA) (**4**). This compound, which is a catabolite of the plant growth hormone (3-indolyl)acetic acid (IAA),[13,14] has indeed been reported to undergo acid-catalyzed ring enlargement to give **3** *via* an opening/ reclosure mechanism.[11] We have prepared racemic compound **4**,[15] which has the same retention time on the HPLC column as the transient species of the hydrolysate mentioned above, and have confirmed that **4** is the genuine aglycon I.



3 4



5

The aglycon II, a markedly air-sensitive compound, was found to be water soluble and to have the molecular composition $C_{18}H_{16}O_8$. Its UV spectrum was similar to that of the secalosides, and it exhibited a complex bathochromic shift when recorded in 0.1 N
NaOH. The ^{1}H-NMR spectrum showed the presence of two benzene rings with a 1,2,4- and a 1,2,4,5- substitution pattern, respectively. Allylic coupling revealed that an aliphatic -CHCHCH- fragment was connected at both ends to the tetrasubstituted aromatic ring. This suggested an indan skeleton, with the additional trisubstituted aromatic ring being attached to a benzylic position. The structure of 5,6-dihydroxy-3-(4-hydroxy-3-methoxyphenyl)indan-1,2-dicarboxylic acid (**5**) accounts best for these observations and for the observed ^{13}C-NMR spectra. The methoxy group was localized on the basis of long-range couplings. The ease by which compound **5** lost water in the mass spectrometer prior to ionization suggested that the two carboxyl functions were *cis* oriented and, hence, predestined for anhydride formation.
However, none of the spectral parameters provided reliable information as to the relative configuration of the adjacent aromatic side chain.

According to their one- and two-dimensional ^{1}H-NMR spectra including COSY-DQF[16,17] recorded in D_2O at 400 MHz, secalosides A and B share

many structural features. Both were shown to contain the spin systems of aglycon I (**4**) and aglycon II (**5**) in a 1:1 ratio. In addition, each contained one α-glucopyranose and one β-glucopyranose moiety, readily identified by their respective anomeric protons. The third hexose subunit of the glycosides did not show an anomeric proton, and its constitution was not immediately obvious. ^{1}H-NMR spectroscopy revealed, besides these constitutional features, that secalosides A and B interchange in solution. In D_2O the equilibrium was reached within less than 24 h with an A/B ratio of roughly 1.3:1. Initially it was thought that this interchange, which seriously impeded the spectral assignment, might be catalyzed by traces of acid. When the spectra were recorded in a 3:2 mixture of MeOH-d_4/C_5D_5N it was found, however, that the interchange was greatly accelerated by the base, and instead of being slowed down, it reached equilibrium in less than 1 h.

Between the two glycosides, the largest chemical shift differences were observed for the AB proton resonances of an ABM-spin pattern appearing at high field. Due to an H/D exchange, this pattern simplified in D_2O slowly to an AB-appearance at δ 2.22 and 2.68 for secaloside A and at δ 2.42 and 2.48 for secaloside B, respectively. The isotopic exchange was faster than the isomerization of the glycosides. We assigned these resonances to the methylene groupings of the (2-oxo-3- indolyl) acetyl fragment (i.e., aglycon I). The ^{1}H-NMR spectra of the glycosides, recorded in DMSO-d_6, showed a free NH resonance at δ 10.4. Consequently, aglycon I must be bound *via* its carboxylic function to the remainder of the molecule. It was concluded, therefore, that secalosides A and B are esters of (2-ox-3- indolyl)- acetic acid (**1**). They are epimers with respect to the chiral center H-C(3)* of the nitrogen-containing heterocycle, and their mutual interconversion resulted from epimerization at this site. This is probably also true for secalosides C and D (**2**). The large number of partially overlapping signals observed in both the ^{1}H- and ^{13}C-NMR spectra of secalosides A and B, being in equilibrium, greatly hampered the assignment. It was not possible to determine the total molecular connectivity of secalosides A and B on the basis of the work done in water or pyridine/methanol. solution to this structural problem is presented in the following paper in this issue.[8]

Table 1. *In vivo* Biological Activities of T-60 Pollen Extract and Purified Fractions Derived Thereof

extract/compd	dose (μg/mouse)	PCV[a] (μL)	T/C cell growth[b] (fraction of control)
control	0	470	1
T60 batch TMO57	1000	0	0
	500	0	0
	250	43	0.09
	125	270	0.57
1	60	0	0
	40	10	0.02
	20	82	0.17
	10	115	0.24
	5	210	0.45
	2.5	330	0.7
3	20	0	0
	10	0	0
	5	0	0
	2.5	0	0
	1.25	140	0.30
	0.625	290	0.62
4	20	0	0
	10	0	0
	5	0	0
	2.5	0	0
	1.25	75	0.16
	0.625	170	0.36

[a] PCV, packed cell volume. [b] *T/C*, treated *vs* control mice cell growth.

Biological experiments carried out so far with the pollen extract Cernitin T-60 have suggested that the latter can inhibit the growth of various tumor cell lines *in vivo* (S180 sarcoma, Lewis lung cancer, and mammary adenocarcinoma EO771) (Sirotnak, F. M. Unpublished data). Biological evaluation of a mixture of secalosides A and B, using the packed cell volume (PCV) assay, as described in the Experimental Section, has shown that these glycosides are highly active *in vivo.* A 10-day treatment of mice with a dose of about 5-10 µg/animal sufficed to kill 50% of implanted S180 tumor cells in the peritoneal cavity of the animals (Table 1). The racemic aglycon I (**4**) and the artifactual isomeric aglycon I' (**3**) were included in the evaluation. Both are found to exhibit very strong antitumor activity against S180 sarcoma. Whereas the cell growth inhibitory concentration at 50% (IC_{50}) is about 5 µg/mouse for secalosides A and B, it is about 1 µg/mouse or less for the aglycons I and I' (Table 1). Because of the lack of available material, the activity of aglycon II could not be evaluated.

In *in vitro* cytotoxicity assays (L1210 leukemia, S180 sarcoma, KB cells), neither Cernitin T60 pollen extract nor mixtures of secalosides A and B and C and D showed any activity, in spite of the high activities demonstrated *in vivo*. The data are particularly significant with regard to S180 sarcoma, as secalosides are active when given ip to S180-bearing mice. Putative mechanism(s) of action may involve activation of the animal's immune system for the following reasons: (1) when mice were treated with

cyclophosphamide (an inhibitor of cell proliferation) 24 h prior to the implantation of S180 tumor cells, the effect of secalosides was abolished; (2) when secalosides were administered ip together with 2-chloroadenosine (an inhibitor of macrophage activation), no effect was detectable; (3) tumor-transplanted mice strains, immunodeficient in T/B cells (nude, SCID, or beige mice) did not respond to treatment with the secalosides (to be published elsewhere). Perhaps the important observation reported here is that most, if not all, of the biological activity of the secalosides appears to reside in a small moiety of the intact glycosides, i.e., the (2-oxo-3-indolyl)acetyl fragment (**4**) or its tetrahydroquinoline counterpart (**3**). Synthetic samples of the racemic compounds **4** and **3** appear to be as active as the whole intact secaloside pair on a molar basis. A further potentialization of action may be obtained upon attachment of one hexose unit, as predicted from the general gross structure of the glycoside.

Experimental Section

General Experimental Procedures. The ^{1}H-NMR and ^{13}C-NMR spectra of the secalosides and of the aglycons were recorded on a Bruker AMX-400 spectrometer (9.4 T) operating at 400 and 100 MHz, respectively. Mass spectra of glycosides were measured by direct insertion on a MS 50 S instrument (Kratos Ltd, Manchester, England) under conditions of electron impact ionization (70 eV) with a probe temperature raised first to 150 °C for about 1 min and then to 350 °C. Optical rotations were determined on a Perkin-Elmer 241 polarimeter. UV spectra were recorded in water on a Varian DMS-80 spectrophotometer within the wavelength range of 400-200 nm. Methods for the detection of biochemical compounds on paper and thin layer chromatograms were carried out as described in ref 9.

Test Material. Cernitin T-60 was obtained as a commercial preparation from AB Cernelle, S-4320 Vegeholm, Sweden. The water-soluble T-60 fraction (pH 4.0-4.4) contained about 60% of the total rye pollen.

Extraction and Isolation. (cf. Figure 1). A portion (400 mL) of the 25% w/v water-soluble T-60 fraction was extensively dialyzed with distilled water for 48 h at 4°C using Spectra/Por dialysis membrane tubing with a cutoff size of 1000 Da. The diffusate (outside fluid) was changed after 24 h. The “inside bag” content was evaporated to a small volume (100 mL) prior to lyophilization.The weight of this fraction, designated “A1”, and was 26 g. The diffusate was discarded. A second dialysis step was carried out with a Spectra/Por membrane tubing (cutoff 2000 Da). Fraction A1 (26 g) was redissolved in 100 mL of H^2O and dialyzed for 1 week at 4 °C. Diffusates (2 L) were collected every 2 days, evaporated, and lyophilized. The yield of recovered yellowish powder, a fraction designated A1.2, was 6 g. This fraction (6 g) was then loaded on three Sephadex G25 (superfine grade, 4.6 cm x 120 cm) columns equilibrated and developed in distilled water at a flow rate of 20 mL/h at 4 °C; 10 mL fractions, distributed into five distinct zones, were collected and monitored at 280 nm. Fractions under each peak were lyophilized and tested for biological activity (*vide infra*). Material exhibiting *in vivo* activity in mice was contained in peaks 3 and 4. Each peak was rerun on G-25 SF columns (2.6 x 100 cm) under the same elution conditions and yielded four to five well-separated subfractions. Major peak subfractions thus obtained were subjected to the biological activity test. HPLC was performed on a Varian 5000 apparatus using columns packed with a Merck reversed-phase Lichro-Cart C_{18}. The analytical column (100 mm x 4.0 mm i.d.) was operated at 1 mL/min, monitoring at 280 nm. Solvent A was 0.1% aqueous TFA, and solvent B was 0.1% TFA in acetonitrile. A gradient of 5-20% over 20 min followed by a plateau for 5 min at 60% acetonitrile was used. Major peaks were collected in Eppendorf tubes and dried down in a vacuum centrifuge (Savant SVC 200). For preparative runs, purification was achieved with a Macherey-Nagel column (250 mm x 21 mm i.d.) Nucleosil 300 Å 5-μm C_8 particles. A linear gradient (10-48% acetonitrile in 0.1% aqueous
TFA) was applied for 45 min at a flow rate of 5 mL/min.

Chemical Hydrolysis. The major, and separately, the minor epimer of glycoside **1** (secalosides A and B) and the major, and separately, the minor epimer of glycoside **2** (secalosides C and D), ca.1-2 mg each, were hydrolyzed in 1 N HCl (100-200 μL) at 100 °C for periods ranging from 5 min to 6 h in small sealed glass tubes;[18] hydrolyzates were dried down, washed three times with 300 μL of H_2O and finally redissolved in 0.1% aqueous TFA prior to RP-HPLC, MS, and/or NMR analyses. Each glycoside gave the compounds **3** and **5**. Insoluble material was discarded.

Enzymatic Hydrolysis. Glycoside mixtures **1** and **2** were subjected to a series of enzymatic digestions using either exoglycosidases or endoglycosidases, such as α- and *β*-glucosidases, α- and *β*-amylases, α-mannosidase, and α- and *β*-galactosidases, using procedures recommended by the manufacturer (Boehringer Mannheim). Digests were analyzed by RP-HPLC. The enzymatic determination of glucose was carried out by the Gluco-Quant test, according to the ad-hoc procedure (Boehringer Mannheim).

Secaloside A (**1**) (major epimer) was obtained as a grayish amorphous powder: mp 178 -186 °C dec; $[\alpha]_D$ +4.6° (*c*0.6, MeOH); UV (H_2O) λ_{max} 320 (sh), 280, strong end absorption; exhibited comparable ^{1}H-NMR data in MeOH-d_4 (400 MHz) as described in ref 8; FAB-MS *m*/*z* $[M + H]^+$ 1002 ($C_{46}H_{51}NO_{24}$).

Secaloside B (**1**) (minor epimer) was obtained as a grayish amorphous powder: mp 192-201 °C dec; $[\alpha]_D$ +6.8° (*c* 0.4, MeOH); UV (H_2O) λ_{max} 320 (sh), 280, strong end absorption; exhibited comparable ^{1}H-NMR data in MeOH-d_4 (400 MHz) as described in ref 8; FAB-MS *m*/*z* $[M + H]^+$ 1002 ($C_{46}H_{51}NO_{24}$).

2-Oxo-1,2,3,4-tetrahydroquinoline-4 carboxylic acid (**3**) (racemic) was obtained as a colorless powder: mp 218-219 °C (lit.[12] mp 220 °C). The UV, IR, ^{1}H-NMR, ^{13}C-NMR, and MS data were identical with those of an authentic sample (refs 11 and 12).

5,6-Dihydroxy-3-(4-hydroxy-3 methoxyphenyl)- indan-1,2-dicarboxylic acid (**5**) was obtained as a colorless, air-sensitive powder: UV (H_2O) λ_{max} (log ϵ) 282.6 (0.17), 250 (sh), 201 (1.41) nm; ^{1}H-NMR (D_2O, 400 MHz) δ 3.76 (1 H, dd, *J* = 9.6, 8.8 Hz, H-2), 3.80 (3 H, s, OCH_3), 4.42 (1 H, br d, *J* = 8.8 Hz, H-3), 4.76 (1 H, br d, *J* = 9.6 Hz, H-1), 6.41 (1 H, d, *J* ≤ 0.8 Hz), 6.84 (1 H, dd, *J* = 8.2; 1.8 Hz, H-6'), 6.90 (1 H, d, *J* = 8.2 Hz, H-5'), 6.93 (1H, d, *J*) 1.8 Hz, H-2'), 6.97 (1H, s); ^{13}C-NMR (D_2O, 100 MHz) δ 54.1 (d, C-3), 55.0 (d, C-1), 58.6 (q, OMe), 59.6 (d, C-2), 114.3 (d), 115.1 (d), 115.4 (d), 118.2 (d), 124.2 (d), 133.5 (s), 138.8 (s), 141.4 (s), 146.4 (s), 146.5 (s), 147.5 (s), 150.2 (s), 179.2 (-CO_2), 180.1 (-CO_2); EIMS (70 eV) *m*/*z* 342 (70) [M^+ - H_2O], 314 (38), 270 (100), 169 (32); HREIMS *m*/*z* 342.0748 (calcd for [$C_{18}H_{16}O_8$ - H_2O], 342.0739).

Packed Cell Volume (PCV) Assay The measurement of the biological activity of various peak fractions was performed by one of us (F.M.S.) at the Sloan Kettering Cancer Center, New York, NY. Briefly, mice (ca. 20 g) were injected at day 1 with 2-3 x 10^5 cells from S180 sarcoma into the peritoneal cavity. They were then treated at days 3, 5, 7, and 9 with different amounts of the samples to be analyzed. At day 10, mice were sacrificed and weighed. The volume of cells remaining within the peritoneal cavity was determined after 10 000 rpm centrifugation of the collected ascetic fluid, which yielded the percentage of cellular volume (V_c). After draining of ascitic fluid and drying of the peritoneal cavity, mice were reweighed and the mass difference reflected ascite volume (V_a) of mice. PCV volume is defined as $\%V_c$ x V_a. The mean PCV value of treated mice (*T*) was compared to that of control mice (*C*). A ratio *T*/*C* of, e.g., 0.5, IC_{50} (inhibitory concentration at 50%) reflects a cell growth of 50% as compared to control. Data are the average of two experiments using six to seven mice/group (see Table 1).

Acknowledgment

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References and Notes

1. Buck, A. C.; Cox, R.; Reece, R. W. M.; Ebeling, L.; John, A. *Br. J. Urol.* **1990**, *66*, 398-404.

2. Rungendorff, E. W.; Weidner, W.; Ebeling, L.; Buck, A. C. *Br. J. Urol.* **1993**, *71*, 433-438.

3. Habib, F. K.; Ross, M.; Buck, A. C.; Ebeling, L.; Lewenstein, A. *Br. J. Urol*. **1990**, *66*, 393 397.

4. Habib, F. K. In *Benign Prostate Diseases;* Vahlensieck, W.,Rutishauser, G., Eds.; Georg Thieme Verlag: Stuttgart, 1992; pp 120-128.

5. Zhang, X; Habib, F. K.; Ross, M.; Burger, U.; Lewenstein, A.; Rose, K.; Jaton, J. C. *J. Med. Chem.* **1995**, *38*, 735-738.

6. Habib, F. K.; Ross, M.; Lewenstein, A.; Zhang, X.; Jaton, J. C. *Prostate* **1995**, *26*, 133-139.

7. Furusawa, E.; Chou, S. C.; Hirazumi, A.; Melera, A. *PhytotherRes.* **1995**, *9*, 255-259.

8. Jaun, B.; Martinoni, B.; Marazza, F.; Bertolin, M. *J. Nat. Prod.* **1997**, *60*, 361-365.

9. Dawson, R. M. C. *Data for Biochemical Research*, 3rd ed.; Oxford Science Publications, Clarendon Press; Oxford, U.K., 1986; pp 453-501.

10. Verbert, A. *Methods on Glycoconjugates*; Harwood Academic Publishers: Chur, Switzerland, 1995.
11. Elderfield, R. C.; Rembges, H. H. *J. Org. Chem*. **1967**, *32*, 3809- 3811.

12. Schmidt, M.; Ro¨ger, P.; Lingens, F. *Biol. Chem. Hoppe-Seyler* **1991**, *372*, 1015-1020.

13. Iskric, S. *Period. Biol*. **1984**, *86*, 153.

14. Nonhebel, H. M.; Kruse, L. I.; Bandurski, R. S. *J. Biol. Chem.* **1985**, *260*, 12685-12689.

15. Szabo-Pusztay, K.; Szabo, L. *Synthesis* **1979**, 276-277.

16. Croasmun, W. R., Carlson, R. M. K., Eds. *Two-Dimensional NMR Spectroscopy: Applications for Chemists and Biochemists*, 2nd ed.; VCH Publishers: New York, 1994.

17. Martin, G. E.; Zektzer, A. S. *Two-Dimensional NMR Methods for Establishing Molecular Connectivity*; VCH Verlagsgesellschaft: Weinheim, 1988; pp 303-316. (18) Spiro, M. J.; Spiro, R. G. *Anal. Biochem.* **1992**, *204*, 152-157.

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Cyclic Hydroxamic Acid Inhibitors of Prostate Cancer Cell Growth: Selectivity and Structure Activity relationships

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BACKGROUND. Clinical symptoms of prostatitis, prostatodynia, and benign prostatic hyperplasia are relieved by the pollen extract cernilton, and the water-soluble fraction of this extract selectively inhibits growth of some prostate cancer cells. A cyclic hydroxamic acid, DIBOA, has been isolated from this extract and mimics its cell growth-inhibitory properties, but the specificity of DIBOA for inhibition of prostate cell growth has not been reported.
METHODS. The in vivo growth inhibitory effects of DIBOA and nine structurally related compounds on DU-145 prostate cancer cells, MCF-7 breast cancer cells, and COS-7 monkey kidney cells were determined by treatment of the cells with various concentrations of the compounds for 2-6 days.
RESULTS. The compounds exhibited a wide range of potencies, but none of them exhibited selective inhibition of DU-145 cell growth. MCF-7 cells were more sensitive to DIBOA than either DU-145 cells or COS-7 cells. 3,4-dihydroquinoline-2 (1H)-one, compound (4), and 1-hydroxy-6-chloro-3,4-dihydroquinolin-2 (1H)-one, compound (7), selectively inhibited MCF-7 cell growth at a concentration of 10 μg/ml. 1-hydroxy-3,4-dihydroquinolin-2 (1H)-one, compound (3), and compound 7 were the most potent inhibitors of DU-145 cell growth. Treatment of DU-145 cells with 3 (100μg/ml) substantially decreased the number of viable cells within 2 days, and no viable cells remained in the culture by day 4.
CONCLUSIONS. It is unlikely that DIBOA, compound (1), is responsible for the selective growth inhibition of prostate cancer cells by the water-soluble fraction of the pollen extract cernilton. Cell morphology results indicate that the growth-inhibitory effects of DIBOA and structurally related agents on DU-145 cells are due to their ability to cause cell death. *Prostate 34:92-99, 1998.* © 1998 Wiley-Liss, Inc.

KEY WORDS: **hydroxamic acids; cancer; prostate; breast**

Introduction

The development of pharmacological agents for treatment of prostate cancer is a continuing challenge to biomedical research. A cyclic hydroxamic acid, 2,4-dihydroxy-2H, 1, 4-benzoxazin-3 (4H)-one (DIBOA, 1), was reported recently to inhibit growth of the DU-145 human prostate cancer cell line [1,2]. DIBOA (1) was isolated from the water-soluble fraction of the pollen extract, Cernilton, which has been shown to cause symptomatic improvement in patients with benign prostatic hyperplasia, chronic prostatitis, and prostatodynia [3,4].

The water-soluble fraction (T60) of cernilton exhibited striking selectivity for inhibition of the growth of human prostate cancer cells lines when tested in vitro. Larynx, liver, bladder, testis, and breast cancer cell lines were not inhibited by T-60, but the hormone-independent prostate cancer cell line DU-145 was very sensitive to the extract. Two hormone- dependent prostate cancer cell lines were less sensitive than DU-145 to the growth inhibitory effects of T-60 [5]. The two reports of the inhibitory effects of DIBOA (1) on DU-145 did not include data obtained with other cell lines [1,2]. We describe herein an investigation of the effects of DIBOA and several structurally related agents on the growth of DU-145 and two other

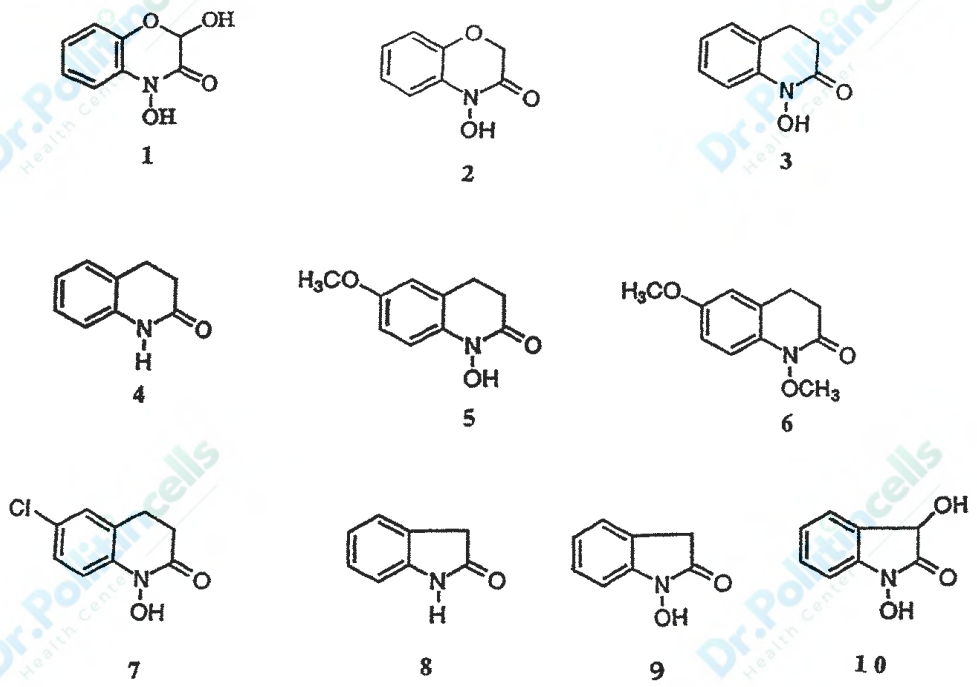

Fig. 1. Chemical structure of DIBOA **(1)** and nine structural analogues evaluated for growth-inhibitory effects in this study. Chemical names: 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one **(1);** 4-hydroxy-2*H*-1,4-benzoxazine-3-one **(2);** 1-hydroxy-3,4-dihydroquinolin-2(1*H*)-one **(3);** 3,4-dihydro-2(1*H*)-quinolinone **(4);** 1-hydroxy-6-methoxy-3,4-dihydroquinoline-2(1*H*)-one **(5);** 1,6-dimethoxy-3,4-dihydroquinolin-2(1*H*)-one **(6);** 1-hydroxy-6-chloro-3,4-dihydroquinolin-2(1*H*)-one **(7);** 1,3-dihydroindol-2-one **(8);** 1-hydroxy-1,3-dihydroindol-2-one **(9);** 1,3-dihydroxy-1,3-dihydroindol-2-one **(10).**

cell lines. The results of this study demonstrate that these compounds are inhibitory not only to prostate cancer cells, but also to MCF-7 breast cancer cells and COS-7 cells. The structure of the agents is shown in Figure 1.

MATERIALS AND METHODS

Chemicals

DIBOA (1) was synthesized according to the published method [6]. Compounds 2-10 were prepared by standard synthetic procedures. The structure and purity of each compound were verified by nuclear magnetic resonance spectrometry, thin layer chromatography, infrared spectroscopy, and elemental analysis.

Cell Lines

DU-145 cells are an androgen-insensitive human prostate cell line derived from a brain metastasis of prostate cancer and were used in this study to model the response of prostate carcinoma cells to hydroxamic acids [7]. MCF-7 cells are a human breast cancer cell line derived from a patient with metastatic mammary carcinoma [8]. COS-7 cells are an SV40- transformed cell line derived from simian CV1 cells [9]. MCF-7 and COS-7 cells were included
to determine the degree of prostate specificity in the action of the hydroxamic acids. DU-145 cells and COS-7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and the MCF-7 cells were a gift from Dr. Norman Sladek, University of Minnesota.

Growth Assays

Cells were plated at a density of 25,000 cells/ well in 24-well plates with RPMI media, supplemented with 4% FBS. Stock solutions of all compounds were prepared in DMSO (10 mg/ml) and dilutions were made so that all cell media received the same DMSO exposure (1%). In control experiments, exposure to 1% DMSO in the culture media was shown to have no effect on cell growth for any of the cell types used in these studies. The compound-containing media were changed every 2 days. Treatments were carried out in triplicate, and each experiment was carried out at least twice. For cell enumeration, cells were trypsinized into a single-cell suspension and counted in an automated Coulter counter (Coulter Electronics, Hialeah, FL).

RESULTS

Time Course and Dose-Response Studies With DIBOA 1

The data shown in Figure 2 represent the effect of a range of concentrations of 1 on three types of cells during a 6-day treatment period. DU-145 cells were sensitive to compound 1 only at the highest concentration of 100 μg/ml (Fig. 2A,A'). Growth inhibition was apparent on the second day and cell growth was only 10% of the control after 6 days. By comparison, the MCF-7 breast cancer cells were much more sensitive to the growth-inhibitory effects of 1 than were the DU-145 human prostate cancer cells (Fig. 2B,B'). MCF-7 cell growth was inhibited moderately by 1 μg/ml of 1, but was markedly inhibited by the higher concentrations of 10 μg/ml and 100 μg/ml. After 6 days, no MCF-7 cells were visible in the wells that had been treated in Figure 3C,C' indicate that the COS-7 cells were more sensitive to 1 than the DU-145 cells , but were less sensitive MCF-7 cells. At both 10 μg/ml and 100 μg/ml, growth of the COS-7 cells was inhibited after 2 days. Similar to the results obtained with the MCF-7 cells, no COS-7 cells were present after 6 days of treatment with 100 μg/ml of compound 1.

Effect of 1 and Structurally Related Analogues on DU-145, MCF-7, and COS-7 Cells

To compare the selectivity and potency of 1 with compounds 2-10 (Fig.1), the three types of cells were treated with two concentrations (10 μg/ml and 100 μg/ml) for a 4-day period as described in Materials and Methods. The results are shown in Figure 3. Treatment of the DU-145 cells with 10 μg/ml of each of the 10 compounds resulted in relatively modest growth inhibition of 10-30% of control values (Fig.3A). Similarly, the inhibition of COS-7 cells was 40% or less under these treatment conditions. Striking differences, however, were observed in the growth-inhibitory potencies of the compounds when MCF-7 cells were treated for 4 days with 10 μg/ml of each agent. DIBOA (1) itself was approximately twice as effective as an inhibitor of MCF-7 cell growth as an inhibitor of either DU-145 or COS-7 cells (Fig. 3A). Compound 4, which is quite structurally dissimilar to 1, caused the same degree of growth inhibition (40%) of MCF-7 cells as 1, and exhibited a similar degree of selectivity for inhibition of MCF-7 growth in comparison to its effects on the other two cell lines (Fig. 3). Compound 7 caused 80% inhibition of the growth of MCF-7 cells at 10μg/ml, but only 17% and 40% inhibition of DU-145 and COS-7 cells, respectively. Thus, compounds 1, 4, and 7 exhibited a selective growth-inhibitory effect on the MCF-7 breast cancer cells. The difference in the effectiveness of 1, 4, and 7 for inhibition of MCF-7 cells in comparison to the other two cell lines was statistically significant ($p<0.05$).

Treatment of the three types of cells for 4 days with 100 μg/ml of compounds 1-10 resulted in a complete loss of selectivity for inhibition of MCF-7 cell growth by 1, 4, and 7 (Fig.3B). At the 100 μg/ml concentration, all 10 compounds inhibited growth of the three cell types, and apparent selectivity was exhibited only by compound 4, which caused 45% inhibition of the growth of DU-145 cells, but 83% inhibition of both MCF-7 and COS-7 cells (Fig. 3B). The weakest inhibitors were compounds 8 and 9.

Growth Inhibition of DU-145 Cells: Dose-Response Comparison

For the purpose of comparing the DU-145 growth inhibition potency of 1 with that of several of its structural analogues, compounds 1-4 and 6-8 were studied at concentrations of 10, 25, 50, and 100 μg/ml over a 4-day treatment period (Fig.4). As shown in Figure 3, compounds 1, 3, and 7 appeared to exhibit similar effectiveness against DU-145 cells at concentrations of 10 and 100 μg/ml. Figure 4, however, illustrates the significantly greater potency of 3 and 7 in comparison to 1, 2, 4, 6, and 8, all of which exhibited similar dose-respond curves. Compounds 3 and 7 inhibited cell growth by 70% and 80%, respectively, at 25 μg/ml, whereas 1 caused less than 10% inhibition at this concentration. The differences in potency were maintained at the higher concentrations of 50 and 100 μg/ml. Thus, both 3 and 7 were more inhibitory to the growth of DU-145 human prostate cells than DIBOA (1). Neither 3 nor 7, however, were selective for DU-145 cells, a characteristic they have in common with compound 1. Indeed, compound 7 appears to be a selective inhibitor of the growth of MCF-7 human breast cancer cells (Fig 3.).

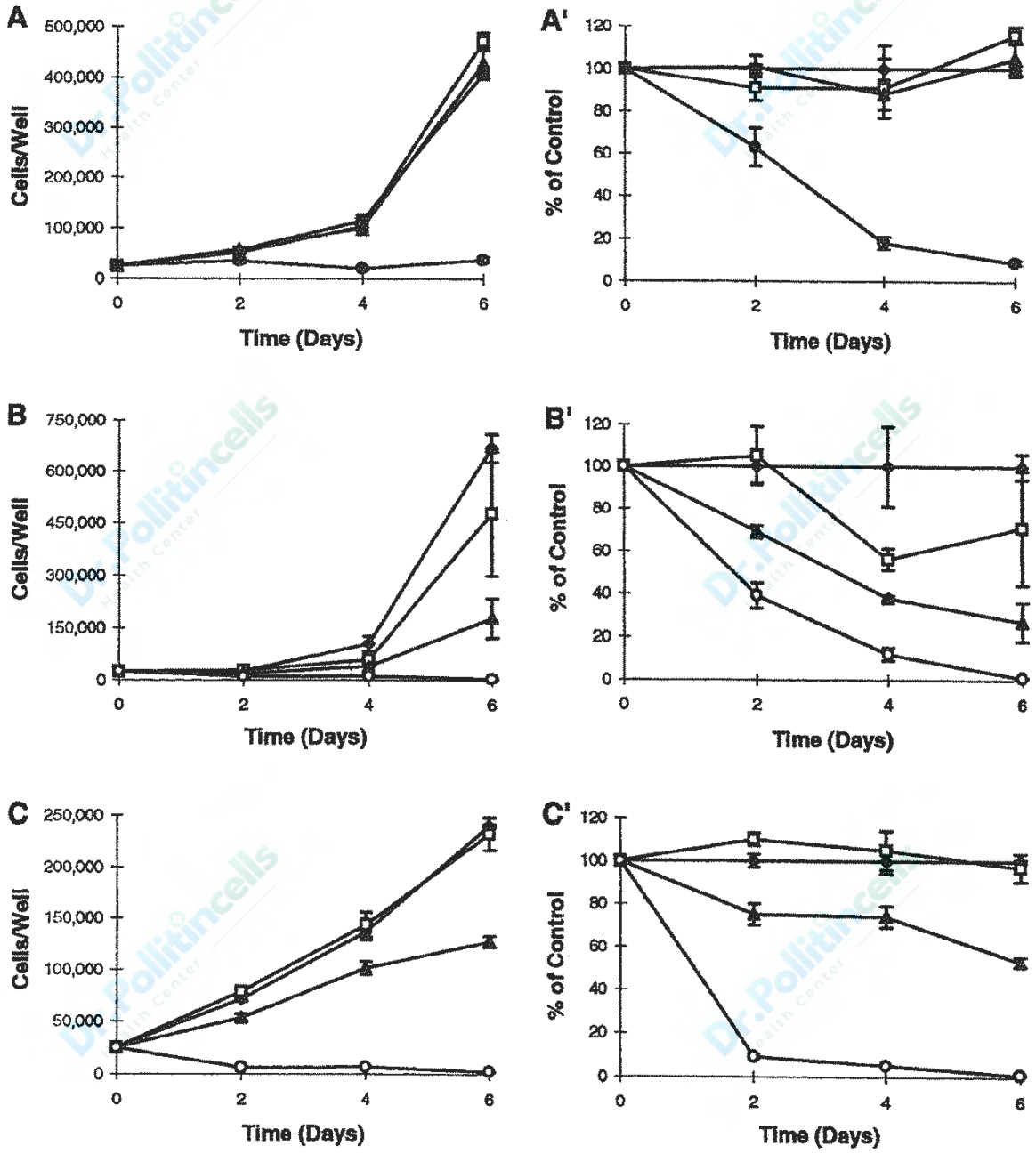

Fig. 2. Effect of compound **1** on the growth of DU-145 cells **(A,A')**, MCF-7 cells **(B,B')**, and COS-7 cells **(C,C')**. Each cell type was treated with 0 (♦), 1 (□), 10 (▲) or 100 (○) μg/ml of compound **1**. Cells were counted 2, 4, and 6 days after treatment was initiated. Data are expressed as total cells per well (A–C) and as percent of control (untreated) cells at each time point (A–C').

Effect of Compounds 1, 3, and 8 on DU-145 Cell Morphology

To characterize the effects of compounds with varying degrees of growth-inhibitory activity on DU-145 cells, morphology was analyzed 2 and 4 days after initiation of treatment with 100 μg/ml of compounds 1, 3, or 8 (Fig. 5). At 2 days of culture the control cells were approaching confluency, and reached confluency by 4 days of culture (Fig. 5A,B0. The control DU-145 cells were relatively small, polygonal-shaped cells that often exhibited cytoplasmic processes extending to make contact with neighboring cells. Numerous lysosomes and lipid droplets populated the cytoplasm, and the nuclei

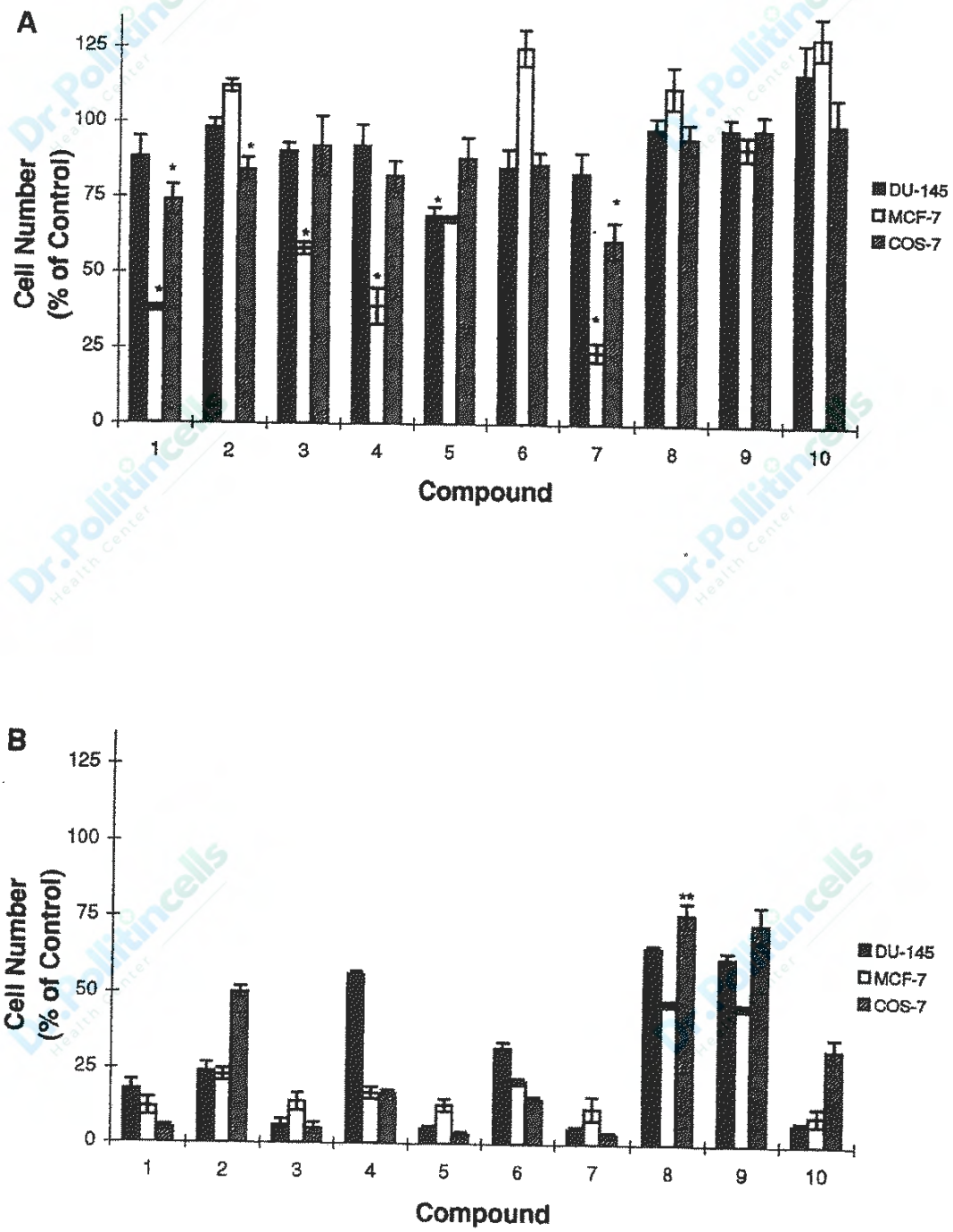

Fig. 3. The growth-inhibitory effect of compounds **1–10** on DU-145, MCF-7, and COS-7 cells. Cells were treated with the compounds at 10 μg/ml **(A)** and 100 μg/ml **(B)** and counted on the fourth day after initiation of treatment. Cell number is expressed as percent of control (untreated) cells. The degree of cell-growth inhibition was assessed for each compound compared to control with Student's *t*-test. *Columns in A that represent a significant growth inhibition compared to control ($P < 0.05$). **Only column in B that does not represent a significant decline in cell growth relative to control.

exhibited prominent nucleoli. The majority of the cells in the sample treated with compound 1 appeared identical to the control cell (Fig. 5C,D). However, at both 2 and 4 days of treatment some cells exhibited degenerative characteristics such as loss of attachment to the substratum and loss of distinct nuclear morphology. At day 4 the cells treated with

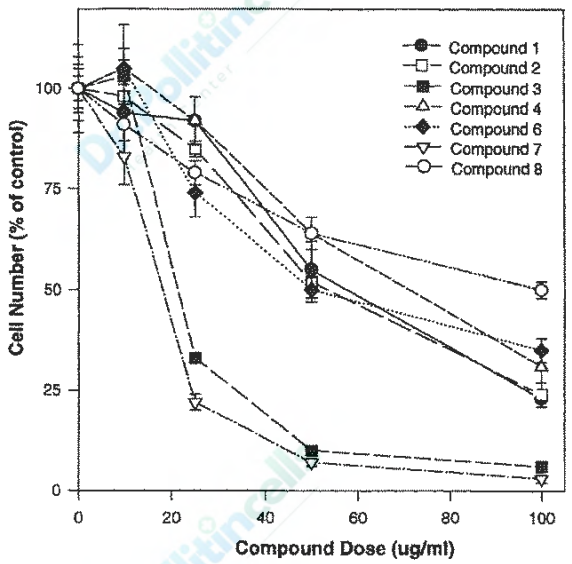

Fig. 4. Growth-inhibitory dose response of representative compounds on DU-145 cells. Cells were treated with 0, 1, 10, 25, 50, or 100 μg/ml of each compound. Cells were counted on day 4.

compound 1 were not yet confluent, consistent with inhibition of cell growth compared to control. Compound 3 had dramatic effects on the DU-145 cells (Fig. 5E,F). On day 2 majority of cells exhibited degenerative changes, but there were still viable cells present (Fig. 5E). The effect of compound 8 on cell morphology was essentially the same as that of compound 1 except that there were fewer degenerating cells, consistent with the less severe effect on cell growth. There was no morphological evidence of cellular differentiation with treatment of any of these compounds.

Discussion

Cernilton, a pollen extract, exhibits clinical effectiveness in the treatment of benign prostatic hyperplasia and chronic prostatitis [3,4]. In vitro studies demonstrate that the relevant biological activity of cernilton resides in the water-soluble cernitin T-60 fraction rather than in the hydrophobic fraction, and that the water-soluble fraction selectively inhibits the growth of DU-145 human prostate cancer cells, but does not inhibit MCF-7 human breast cancer cells [5]. DIBOA (1, Fig.1) was isolated from the water-soluble fraction of cernilton and exhibited growth-inhibitory action on DU-145 cells [1,2]. In contrast to the results reported from studies with the water-soluble fraction, the data shown in Figure 2 indicate that DIBOA (1) does not selectively inhibit the growth of DU-145 cells, but is rather a more potent inhibitor of the growth of MCF-7 human breast cancer cells. Thus, the reported selectivity of the water-soluble cernitin T-60 fraction for inhibition of DU-145 cells is unlikely to be attributable to the action of DIBOA (1). Further, compound 1 effected inhibition of the growth of MCF-7 and COS-7 cells at 10 μg/ml, a concentration which did not slow the growth of DU-145 cells. Thus, in the present studies, 1 was found to be a more effective growth inhibitor of both MCF-7 and COS-7 cells than of DU-145 cells and, at a concentration of 10 μg/ml, exhibited selectivity for MCF-7 cells (Fig. 3). The selectivity was lost when a concentration of 100 μg/ml of 1 was used (Fig.3). Although, this study was not designed to determine the mechanism whereby these hydroxamic acids inhibit cell growth, it is evident from the cell morphology data that DU-145 cells are killed by these compounds, and the extent of cell death seems to correlate with the degree of cell-growth inhibition. Whether or not the compounds also have an effect on the kinetics of cell division cannot be determined from these experiments.

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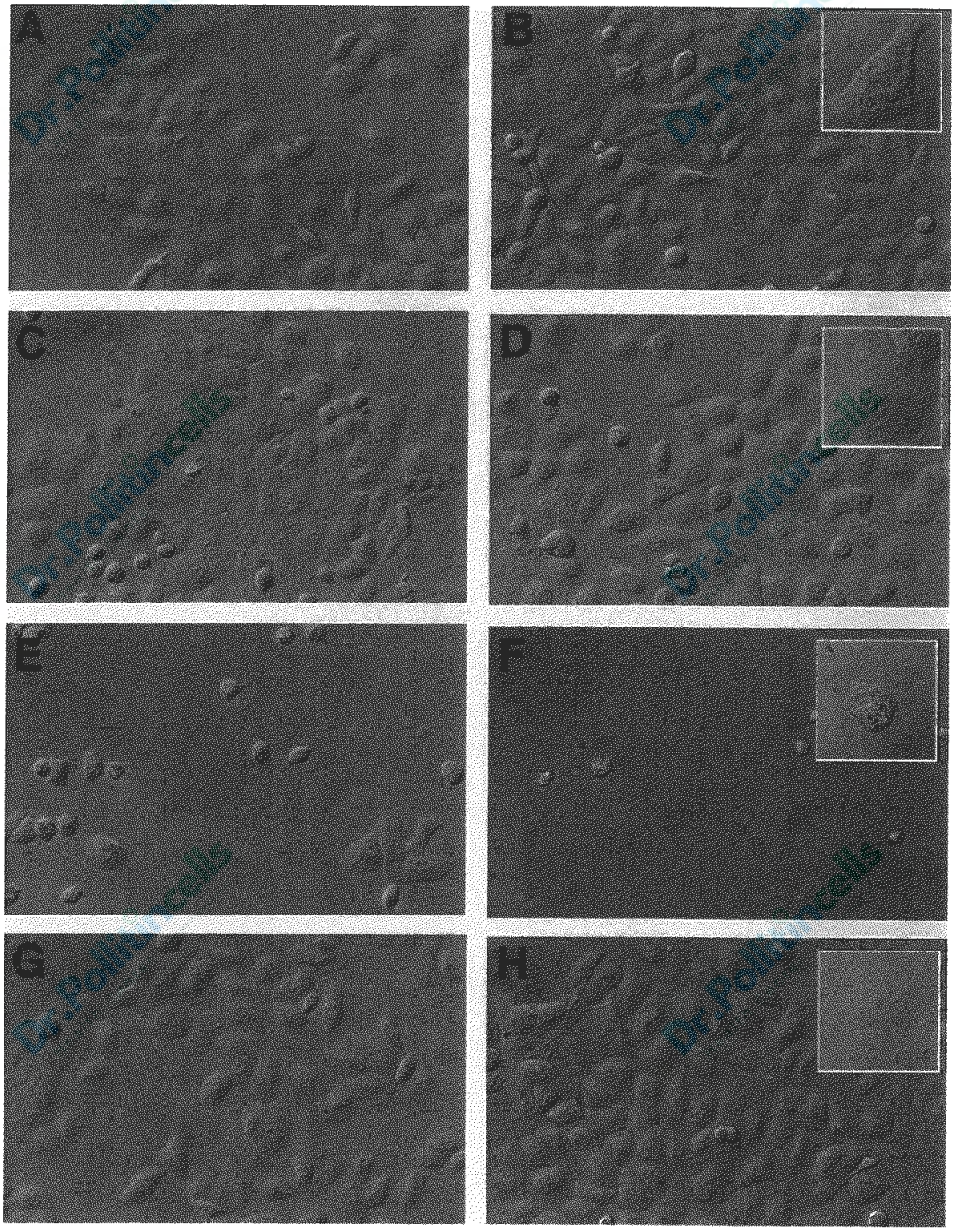


Figure 5.

Fig. 5. Effect of compounds **1, 3,** and **8** on DU-145 morphology. DU-145 cells were plated on 8-well microscope slides at the same density used in the cell growth assays. Cells were allowed to attach to the microscope slide for 24 h before treatment with a compound. At 2 and 4 days after initiation of treatment, cell morphology was analyzed by Nomarski differential-interference-contrast microscopy. Cells were viewed in culture media, without fixation, under coverslips. **A, C, E, G:** Cell morphology recorded on day 2. **B, D, F, H:** Cell morphology on day 4. Cells in A and B were not treated with any compound (control); cells in C and D were treated with 100 μg/ml of compound **1;** cells in E and F were treated with 100 μg/ml of compound **3;** and cells in G and H were treated with 100 μg/ml of compound **8.** Magnification in general is $600_{\times}$, while magnification of insets in B, D, F, and H is $1,800_{\times}$. Cells treated with compound **1** begin to exhibit degenerative morphology (irregular shape and detachment from the substratum) and a noticeable decrease in cell number by day 4 (D). Note the dramatic effect of compound **3** on cell morphology, as well as cell number, at both 2 and 4 days (E and F, respectively). The effect of compound **8** on cell morphology is intermediate between that of compound **1** and control.

One objective of this study was to obtain information about the structural requirements for inhibition of the growth of DU-145 cells by 1. The hydroxyl group in the 2-position of compound 1 renders the compound capable of undergoing ring opening under aqueous conditions to generate, successively, an α-keto aldehyde and an isocyanate [10]. Both of the latter are reactive chemical species that can form covalent adducts with cellular constituents and contribute to inhibition of cell growth. Compound 2, however, which does not contain a 2-hydroxyl group and cannot undergo ring opening (Fig.1), was approximately equal in potency to 1 as an inhibitor of DU-145 growth (Fig.4). Further, compounds 3 and 7, which contain neither the 2-hydroxyl group nor the 1-oxygen atom of 1, were both more potent inhibitors of the growth of DU-145 cells than was 1 (Fig.4). Thus, if compounds 1-3 and 7 inhibit cell growth by a common molecular mechanism, the mechanism does not involve generation of reactive α-keto aldehydes or isocyanates.

Cyclic hydroxamic acid analogues of 4 have been reported to exhibit antimicrobial activity, and compound 1, but not 4, was mutagenic to *Salmonella typhimurium* TA98 and TA100 [10-12]. Thus, the growth-inhibitory effects of such agents are not unexpected. An unanticipated result of the present study, however, was the relatively potent and selective growth-inhibitory effect of compound 7 on MCF-7 human breast cancer cells (Fig. 3). Compounds 1 and 4 also exhibited selectivity for inhibition of MCF-7 cells and are not close structural analogues of 7. Compounds 1, 4, and 7 may warrant further investigation of their inhibitory actions and breast cancer cells.

References

1. Zhang X, Habib FK, Ross M, Burger U, Lewwnstein A, Rose K, Jaton J-C: Isolation and characterization of a cyclic hydroxamic acid from a pollen extract, which inhibits cancerous cell growth *in vitro*. J. Med. Chem. 1995; 38: 735-738.
2. Habib FK, Ross M, Lewenstein A, Zhang X, Jaton J-C: Identification of a prostate inhibitory substance in a pollen extract. Prostate 1995; 26: 113-139.
3. Buck AC, Cox R, Rees RWM, Ebeling L, John A: Treatment of outflow tract obstruction due to bengin prostatic hyperplasia with the pollen extract, Cernilton. Br J Urol 1990; 66: 398-404.
4. Rugendroff EW, Weidner W, Ebeling L, Buck AC: Results of treatment with pollen extract (Cernilton® N) in chronic prostatitis and prostatodynia. Br J Urol 1993; 71: 433-438.
5. Habib RK, Ross M, Buck AC, Ebeling L, Lewenstein A: In vitro evaluation of the pollen extract, Cernitin T-60, in the regulation of prostate cell growth. Br J Urol 1990; 66: 393-397.
6. Sticker D, Pratorius B, Mann G, Meyer L: A convenient synthesis of 2, 4-dihydroxy-2H-1, 4-benzoxazin-3 (4H)-one. Synthesis 1989; 211-212.
7. Stone KR, Mickey DD, Wunderli H, Mickey Gh, Paulson DF: Isolation of a human prostate carcinoma cell line (DU 145). Int J Cancer 1978; 21: 274-281.
8. Soule HD, Vazquez J, Long A, Albert S, Brennan M: A human cell line from a pleural effusion derived from a breast carcinoma. J Natl. Cancer Inst. 1973; 51: 1409-1413.
9. Gluzman Y: SV40-transformed simian cells support the replication of early SV40 mutants. Cell 1981; 23: 175-182.
10. Atkinson J, Morand P, Arnason JT, Niemeyer HM, Bravo HR: Analogues of the cyclic hydroxamic acid 2, 4-dihydroxy-7-methoxy-2H-1, 4-benzoxazine-3-one: Decomposition to benzoxazolinones and reaction with ß-mercaptoethanol. J Org. Chem. 1991; 56: 1788-1800.
11. Davis Al, Hulme Kl, Wilson GT, McCord TJ: *In vitro* antimicrobial activity of some cyclic hydroxamic acids and related lactams. Antimicrob Agents Chemother 1978; 13: 542-544.
12. Hashimoto Y, Shudo K, Okamoto T: Mutagenicities of 4-hydroxy-1, 4-benzoxazinones naturally occurring in maize plants and of related compounds. Mutat. Res. 1979; 66: 191-194.

New immunomodulators with antitumoral properties; Isolation of active naturally-occurring anti-mitotic components of MR>1KD from pollen extract T60

Jean-Claude Jaton, Geneva, April 1994

The *in vivo* assay for inhibitory active components present in a pollen extract served as an efficient guide-line for the purification procedure outlined below (Table 1). The designation of components listed in Table will be fully described in the text and figures. Briefly, the active material exhibits a size higher than 1 kDa but lower than 2 kDa; such material is comprised within Sephadex G-25 SF fraction III. The most active component appeared to be fraction III-b5, as summarized in Table 1. The final purification step was carried out by HPLC on a reverse phase column (molecules b51/b52). Two approaches were attempted.

A. From T60 SF-011 (containing up to 60% maltodextrine)

1. Dialysis of T60 SF-011 vs. dist. water for 48 hr using Spectra/ Por membrane MW CO 1,000

Dissolve 80 g of T60 in 280 ml distilled water. Fill Spectra/Por molecularporous membrane tubing MW CO 1,000 with T60 solution. Fill up to 1/3 of the tubing content. Length of tubing about 45 cm. Six tubings should be prepared. Dialyse vs. 101 H_20 in the cold room (+ 4°C) and change the diffusate (outside fluid) after 24 hr. After 48 hr, remove the bags from the diffusate, open each one and concentrate the "inside bag" content (brown color) to a small volume (100-150 ml) prior to lyophilization. Do not remove the precipitate.

Recovery: on the average, 20 g, i.e. ~25% ~20g A1

This preparation is called A1.

2. Dialysis of "A1" using Spectra/Por membrane MW CO 2,000 vs. water

In this step, the active components are to be found in the diffusate ("outside fluid") since the molecular weight cut off value is 2 kDa (MW CO 2,000). Dissolve 20g "A1" in 100 ml H_20 and distribute the solution (with precipitate) into 4 tubings: each tubing should be filled up to 1/3 of its total content. Dialyse 4 bags vs. 2 1 H_20 (i.e. 2 bags/ 1 l-cylinder) at 4°C for one week. Pool the diffusates from both cylinders every day (21), evaporate (Büchi Rotavapor) to 100 ml and lyophilize.

After one week: the yield of recovered material (slightly yellowish powder) is about 25-30%. This material is designated "A2". 5-6g

Then, the A2 mixture is subjected to Sephadex G-25 SF gel filtration in order to separate active fractions IIIa, IIIb, and IIIc (Fig. 1).

Fraction IIIb was further rerun on a similar gel filtration column and 5 subfractions were obtained, of which III-b5 was found to be the major one (Fig. 2).

Fraction III-b5 was finally purified by H.P.L.C. and 2 major fractions, designated III-b51 and III-b52 were obtained. Typical data are shown for information (Fig 3).

The ratio of b51 to b52 in fraction III-b5 is about 61.7% to 38.2%, respectively, as measured from the area under each peak detected at 280 nm. Fractions b51 + b52 account for about 40% of the total absorbing material at 280 nm.

B. FROM SPISSUM (without maltodextrine).

Dialysis of spissum TA 080 code N° 207000-17

Expected content: 23 g % (i.e. 115 g/ 500 ml)

Volume: 500 ml

1. First dialysis using Spectrapor membrane CO 1 kDa vs. 10 liters of water.

Eight castings were prepared and filled up to one third of the total volume with undiluted visqueous spissum solution. Dialysis time was 48 hr with 4 changes of water. The content "inside bag" was recovered and lyophilized : 16 g.

Recovery $^{16}/_{115}$ ≈ 14%

This material is designated A1 from Spissum

2. Second dialysis of A1 from spissum using Spectrapor membrane CO 3.5 kDa vs. water

A1 (16 g) was dissolved in 40 ml H_20 and dialyzed vs. 1 liter of water for 24 hr. The outside fluid was changed every day for a period of up to 9 daysin the cold room. The dialysate was evaporated to a small volume and lyophilized every day or every 2 days.

Recovery ("outside bag" content)	Day 1	600 mg
	2	500 mg
	3	250 mg
	4 + 5	400 mg
	6 + 7	200 mg
	8 + 9	200 mg
	TOTAL	2'150 mg

Yield (after 9 days of dialysis) : ~13%

This material was designated A 3.5.

Seperation of 2g of A 3.5 on Sephadex G-25 SF (2.6 cm x 90 cm)

Conditions: as usual

Recovery of fractions:

IIIa : 45 mg

IIIb : 39 mg
IIIc : 22mg

Seperation of fraction III-b on Sephadex G-25 SF

See Fig. 2 for details, which shows the separation into FrIII-b1, b2, b3, b4, and b5; these fractions were derived from T60 SF-O11. Fig. 4 shows similar data but obtained from material derived from spissum TA 080. Fraction b5 is the prominent fraction (80%).

Fractionation of fraction III-b5 on HPLC

Two major fractions, designated b51 and b52 were routinely obtained, either from T60 SF11 (Fig. 3) or directly from spissum TA 080, as illustrated in Fig. 4.

Varian HPLC program

Solvent A 0.1% TFA

Solvent B acetonitrile (ACN)

T (time)	
0 min	5% B (ACN)
20 min	20% B (ACN)
21 min	60% B (ACN)
24 min	60% B (ACN)
25 min	5% B (ACN)

Equilibration time : 10 min

Injection- to injection time : 35 min.

Molecules b51 and b52 may be epimers in solution

Preliminary data from Prof. U. Burger (Univ. of Geneva) suggest that b51 and b52 are interconvertible, as suggested by NMR spectra in D_2O after a few days at 4°C. On the other hand, mass spectrometry spectra of b51 and b52 were virtually identical. Yet, b51 and b52 were separately isolated from preparative HPLC runs and lyophilized. The separation efficiency was better than 98%. An aliquot of b51(in D_2O), and of b52(in D_2O) was injected into HPLC, respectively. The chromatograms are presented in Fig. 5 & Fig. 6.

Conclusions:

1. purified b51 (one single peak) converts into b52 and reciprocally. Rentetion times of both compounds are identical to those of b51 and b52 present in fraction III-b5 (i.e. in pollen extract).
2. minor peaks at 12 min, and the doublet peak around 17 min may reflect degradation products from b51 and b52, which are also visible in fraction III-b5(Fig. 3).
3. the ratio of purified b51, which converts into b52 (Fig. 5) is:

b51 (63.8%) $\longrightarrow$ b52 (36.2%)

4. conversely, the ratio of purified b52, which converts into b51 (Fig. 6) is:

b52 (40%) $\longrightarrow$ b51 (60%).

Thus, b51 and b52 are likely to be present in an equilibrium in pollen extract, i.e. about 60% b51/ 40% b52. Isolated species (either b51 or b52) interconvert and yield the same ratio, as found in pollen extract.

Biologically active molecules from the b series are likely to be glyconjugates.

Microchemical determinations of G-25 SF fraction b5, and of fractions b51/b52 highly suggest that they contain sugar units and an aglucone moiety, which behaves as a phenolic compound. This was based on data which were obtained upon mild hydolosis of b5 or b51/b52 at 100°C for 6 hr in the presence of bidistilled 1.0 N HC1 under high vacuo. TLC patterns of the hydrolyzate unraveled monosaccharide (glucose) and an aglucone, which positively stained with the Pauli reagent. Because rough MS data suggested an atomic mass of 1002.2 kDa for both b51 and b2, the expected composition ob b51/b52 could be aglucone moiety linked to a tetra- or penta-glucose unit. The type of glycosidic linkage (α-or β-) was not evaluated.

The data should be taken with caution, as no high resolution mass spectrometry nor NMR spectra were yet determined for the aglucone. Runs in a 600 mHz NMR machine in Zurich may be helpful for the identification of the sugar moiety, in particular about the α- or β-glycosidic linkages [Prof. U. Burger, March, 1993]. Mass spectrometry data should be available before Eastern 1993 (Prof. J.-C. Jaton, Dr. K. Rose, Dept. of Medical Biochemistry).

Hydolysis of b51/b52 molecules

The method used was based on the recent work of Spiro & Spiro (Anal. Biochem. (1992) 204, 152-157). Conditions were: 3-5 mg of glycoconjugate in 250-330 µl of 1.0 N HCl in a glass hydrolysis tube. Temperature: 100°C for 6 hr under vacuo. Drying down step followed by washing with water and centrifugation of dark brown precipitate. Supernatants were kept at 4°C prior to HPLC fractionation.

HPLC analysis of hydrolyzate from b51/b52

A. <u>Under no vacuum</u>

With a mixture b51/b52 (60%/40%), major peaks 2 and 4 were recorded according to Fig. 7. Hydolysis products will be designated by H.

First run peak 2 b51H	Elution time : 16.45
2nd run peak 2	" " : 16.26
First run peak 4 b52H	Elution time : 17.46
2nd run peak 4	" " : 17.24

B. Under vacuum

Hydrolysis of b52 compound only; detection at 295 nm rather than at 280 nm. See Fig. 8.

Peak 1 = b51H	elution time : 16.16	28% (OD_{295})
Peak 2 = b52H	elution time : 17.69	72% (OD_{295})

Preliminary characterization of biomolecules present in fraction III-a

Pooled fraction III-a was loaded onto a (2.6 x 90 cm) Sephadex G-25 SF column. Two major peaks are apparent and the material under each peak was collected. From 140 mg III-a, a3 accounts for 30 mg and a4, 66 mg (Fig. 9). Analytical runs of fraction a3 and a4 were carried out (Figs. 10 & 11) by HPLC under standard conditions.

Material a3 discloses 3 major fractions, designated A31, A32, and A33. The last eluting peak at 25.63 min is of no interest, because of the washing of the column (Fig. 10). Material a4 is more complex with possibly 3 pairs of compounds (Fig. 11):

a) the pair eluting at 17.71 min + 18.02 min.

b) the major eluting at 18.76 min + 19.51 min.

c) the pair eluting at 20.58 min + 21.77 min.

The last pair (c) may well be the b51/b52 pair (see Fig. 3 for retention times) as fraction a is cross-contaminated by b (Fig. 1).

Preliminary MS data obtained from HPLC purified compounds a31 and a33 (Fig. 10.), and a41 and a42, respectively (Fig. 11).

Disappointing MS spectra (electrospray) were recorded for a31 and a33.

a31: signals at 597/575/439 are the major ones. A minor signal at 940.

a33: major signals at 801/815 and lower masses data.

Conclusions: strange, do not make sense at the moment.

Better, encouraging MS spectra were obtained for a41 and a42.

a41: 1164; 1183; 1002.2; 840; 822

a42: 1164; 1182.9; 1002.1; 840; 822

A41 and a42 appear to exhibit the same MS spectra.

Conclusions:

a) a41/a42 constitute probably a pair similar to the pair b51/b52

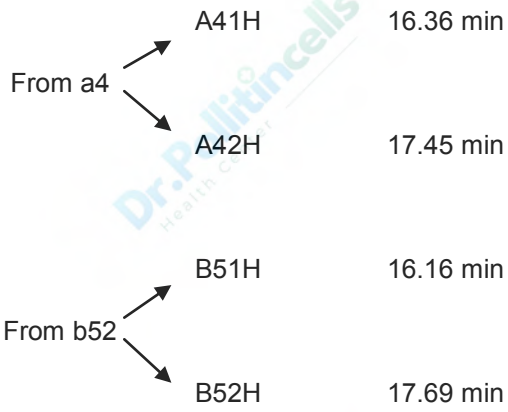
b) the MS spectrum of a41/a42 pair also exhibits degradative material of Mr 1002.2 kDa, i.e. precisely the mass of the pair b51/b52; when one more hexose unit is removed from a41/a42,

one should get 840.2 (1002 -162). If one hydrated hexose unit is removed, one should get 822, which is consistent with what we observed. Thus, I feel that the pair a41/a42 differs from the pair b51/b52 by the addition of one more hexose unit to the b51/b52, yielding a41/a42. Furthermore, hydrolysis results suggest that the aglucone might be the same in the a and b series (see below).

Hydolyzate of a4 (crude a4 according to Fig. 11)

The results are illustrated in Fig. 12, which shows that peak A41H and A42H are present in the same ratio (36% vs. 64%) as found for the hydrolyzate of b51/b52 and that the retention times of A41H + A42H are virtually identical to those of the hydrolysis products of b51/b52.

Retention times from hydrolysis products of a4 and of b52



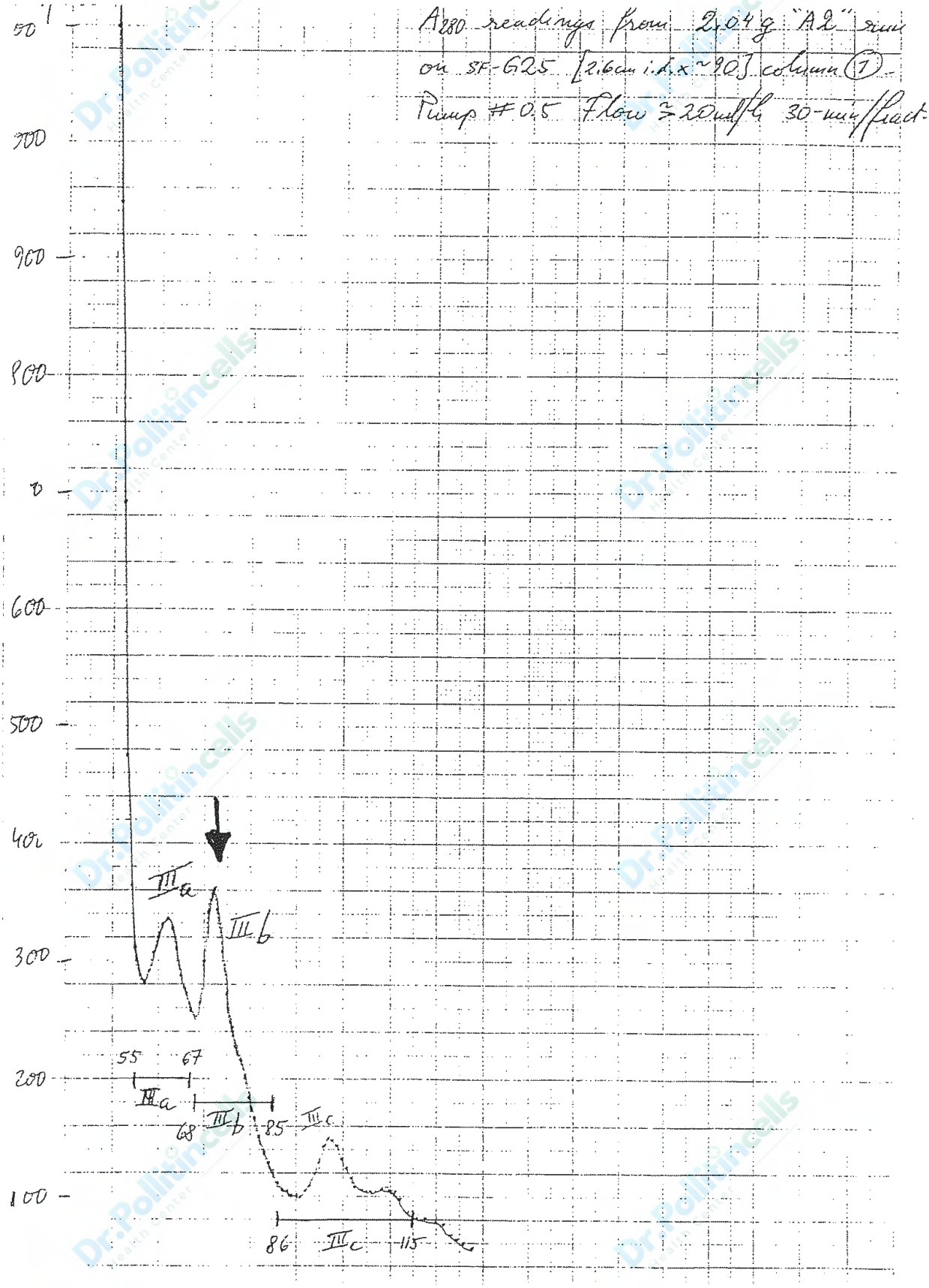
The hydrolyzates from a4 and b52 will be subjected to MS spectroscopy and NMR analyses. The material (~1 mg) was provided on March 29-31, 1993 to Dr. K. Rose (MS) and to Prof. U. Burger (NMR, Sciences II). Very preliminary data from Prof. Burger suggest that that protonic NMR spectra of the hydrolysis products from a41/a42 and b51/b52 are indistinguishable. Thus, the aglucone of a or b is likely to be the same.

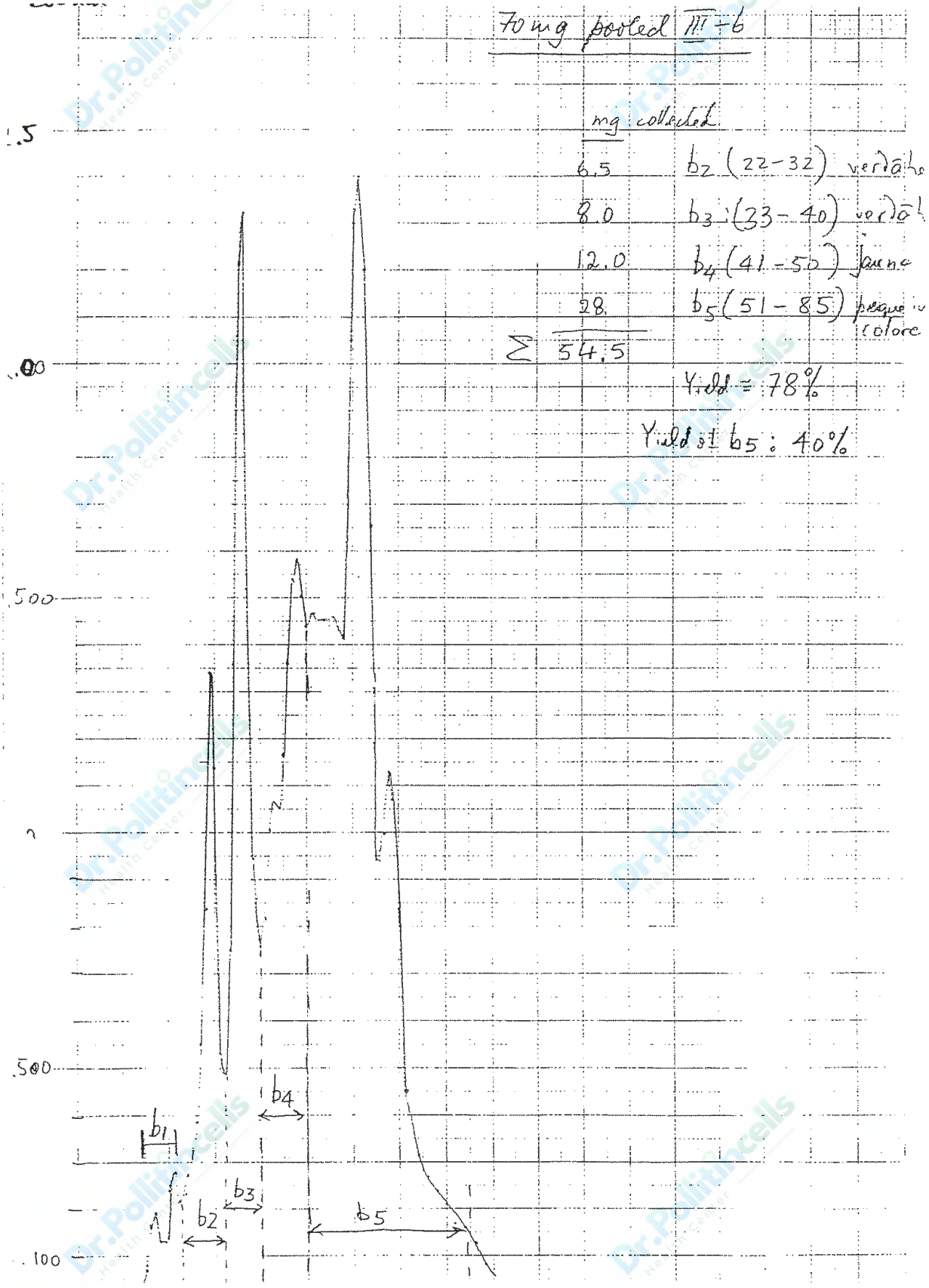
We can, at that time, speculate that biomolecules of the b series (b3, b4 and b5) or of the a series (a3, a4, and a5), which display significant inhibitory activity (Table 1), are all related to each other; they may well differ from each other by the number of hexose units attached to the aglucone moiety.

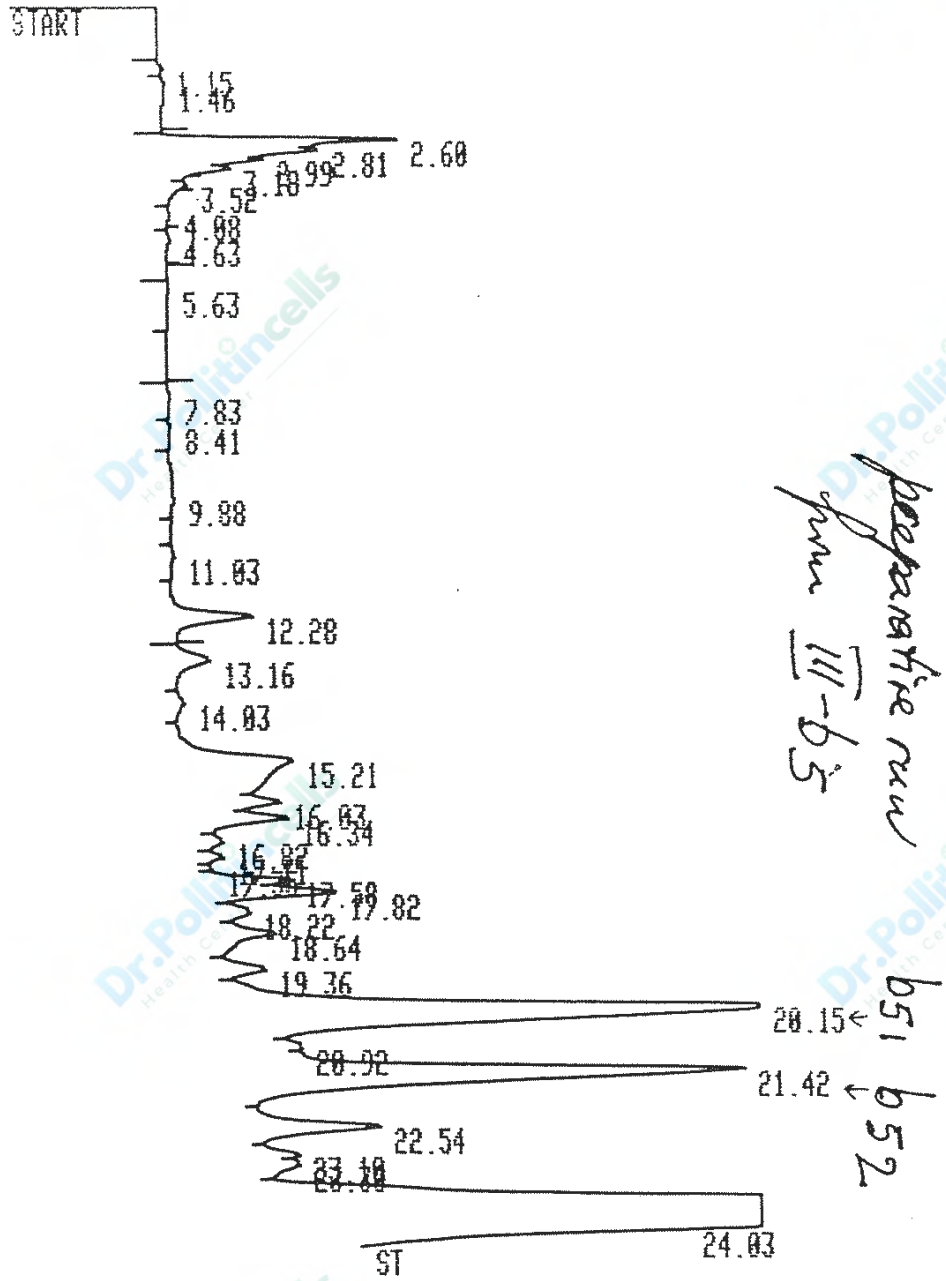
Table 1. *In vitro* bioassay for inhibitory naturally-occurring components from a pollen extract.

As developed by Prof. Sirotnak at the Memorial Sloan Kettering Cancer Center in New York. S180 tumoral cells were injected in the peritoneal cavity of a group of 10 mince (ascite formation); following implantation, mice received the drug to be tested i.p.; after one week, the volume of packed cells is measured, thus giving the % growth of tumor as compared to control. The assay is referred to as the "packed cell volume assay."

	Compounds	IC50 (mg or μg/mouse)	
I	SF-011 T60	1.6 mg	
	"A1"	0.39 mg + 0.5 mg	
	G-25 SF fraction III (a + b + c combined)	< 50 μg	
II	SF-011 T60	2.5 mg + 0.5 mg	
	III-a3	< 12.5 μg	← weight loss !
	a4	25 μg	
	a5	> 50 μg	
	III-b3	15 μg	
	b4	18 μg	
	b5	< 12.5 μg	
III	T60 SF-011	2.8 mg	
	III-b51 (HPLC purified)	6.5 μg	
	III-b52 (" ")	> 20 μg	


A280 readings from 2.04 g "A2" run
on SF-625 [2.6cm i.d. x ~30] column (I)
Pump #05 Flow ≈ 20ml/h 30-min/fract.
50
700
900
800
0
600
500
400
300
200
100
III a
III b
55
67
III a
68
III b
85
III c
86
III c
115


70 mg pooled III-6
mg collected
6.5 b2 (22-32) verdāle
8.0 b3 (33-40) verdāle
12.0 b4 (41-50) Jaune
28. b5 (51-85) beige
Σ 54.5
Yield = 78%
Yield of b5 : 40%
b1
b2
b3
b4
b5

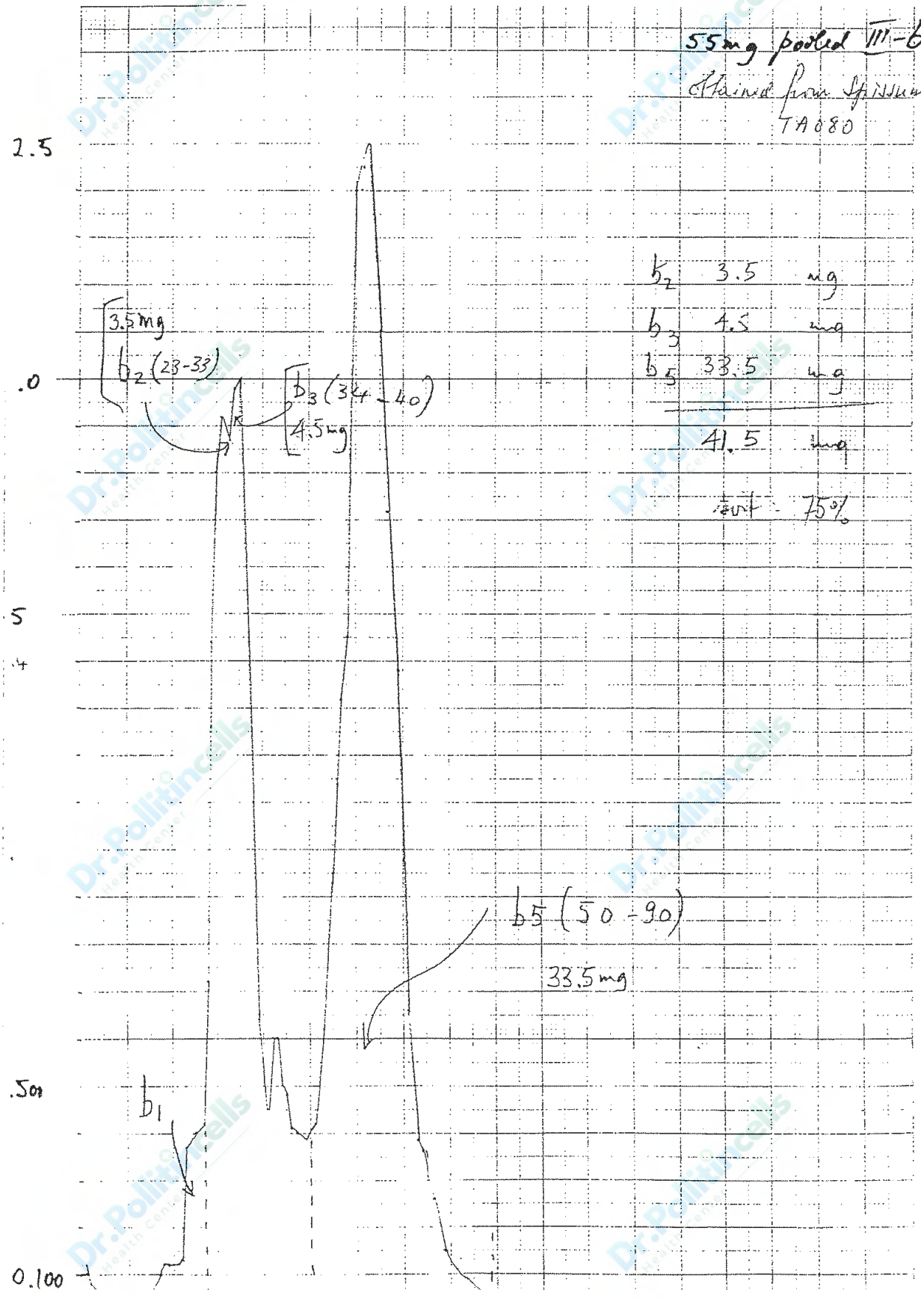


preparative run
fraction III-b5

b51 b52

HPLC profile of III-b5
detection uv at 280 nm

Fig. 3

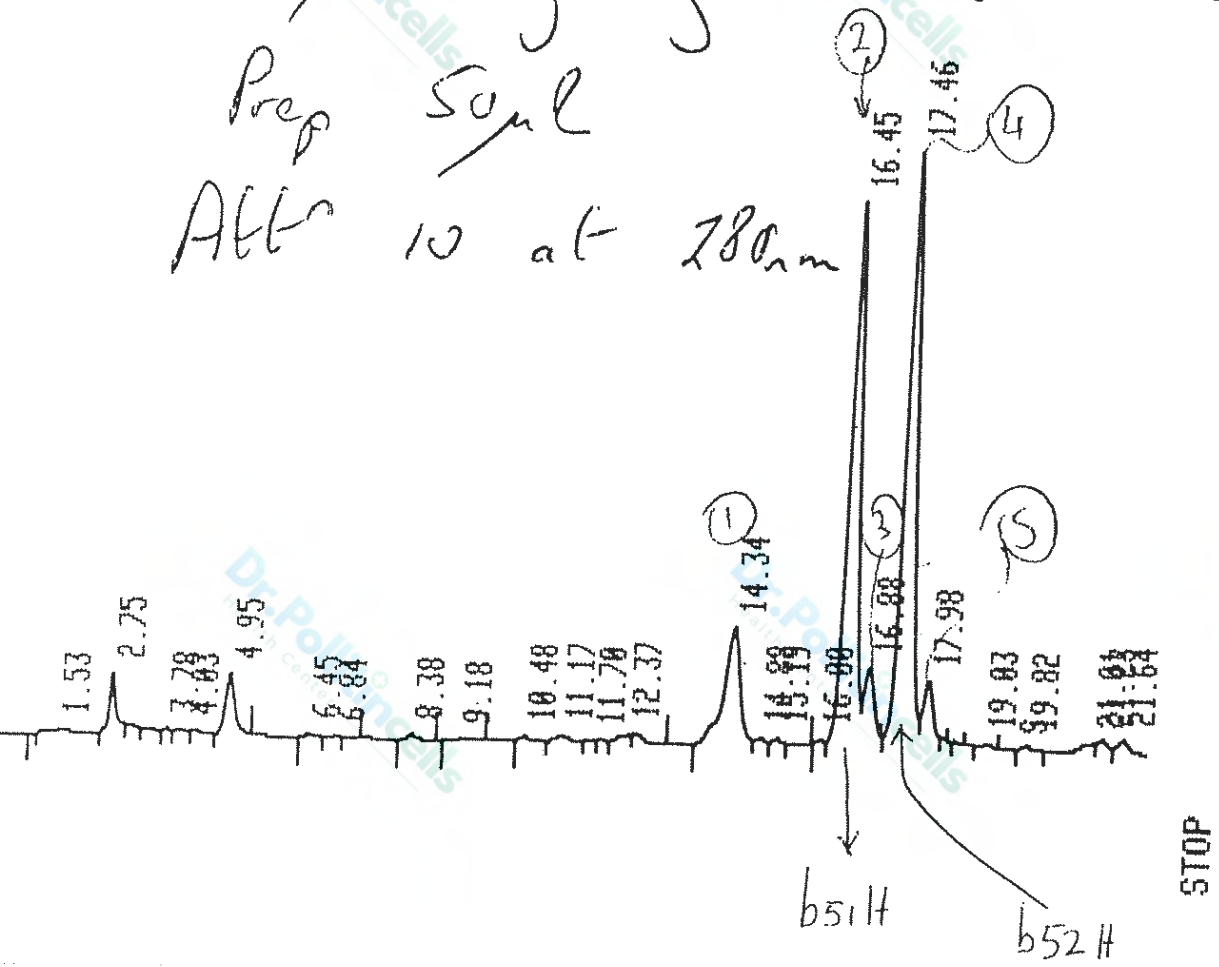

55mg pooled III-6
Obtained from Hirudo
TA080
2.5
.0
5
.50
0.100
3.5mg
b2 (23-33)
b3 (34-40)
4.5mg
b5 (50-90)
33.5mg
b1
b2 3.5 mg
b3 4.5 mg
b5 33.5 mg
41.5 mg
Yield: 75%

HILC profile of pure b52 ret. 2 wk later
on the same HPLC column → 2 forms in equilibrium
b52 20µl D2O (tube RMN)
START
1.57
3.55
12.62
17.96
20.45
21.63
23.71
ST

50µl hydrolyzed (b51+b52) under no vacuum

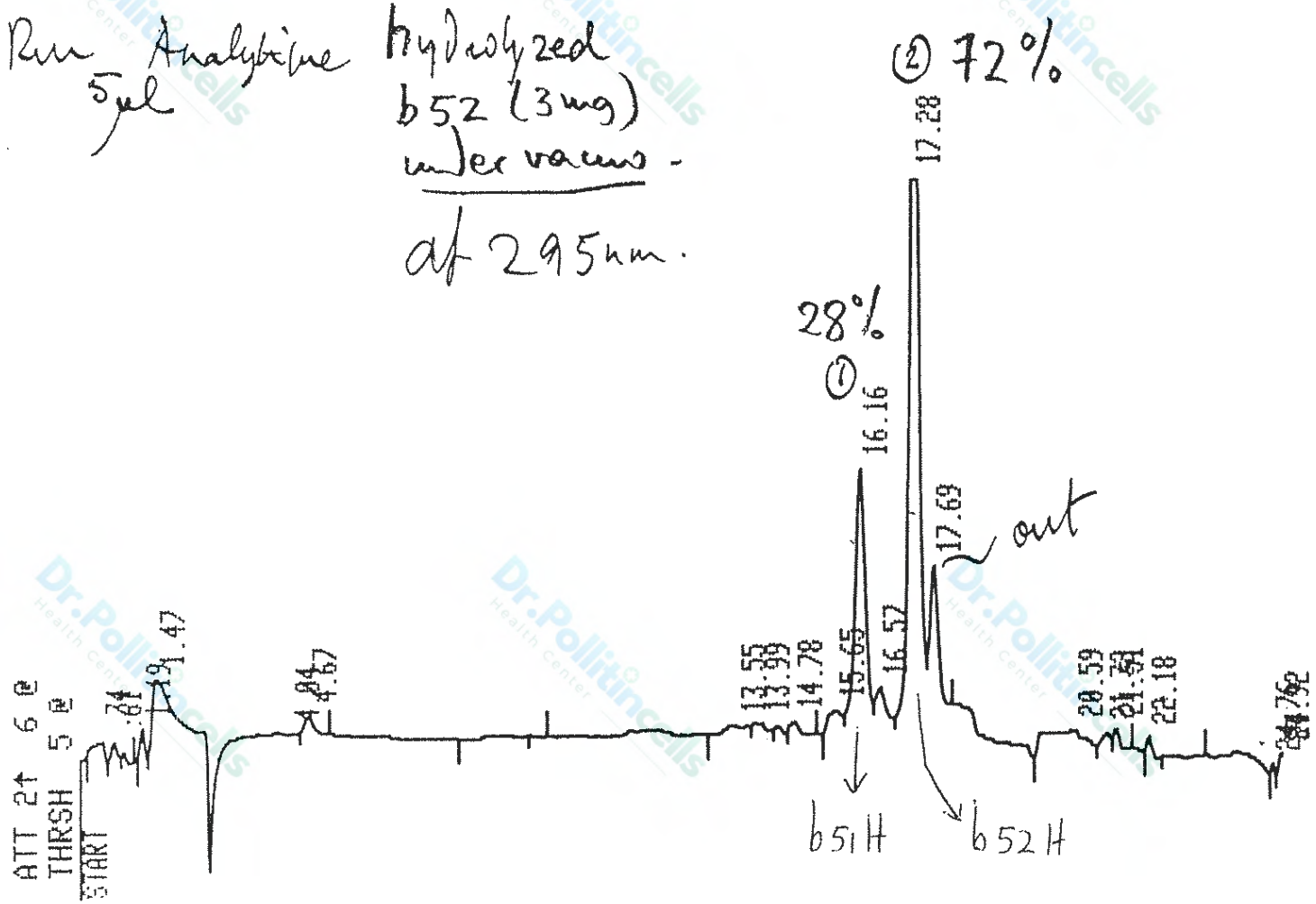
Prep 50µl

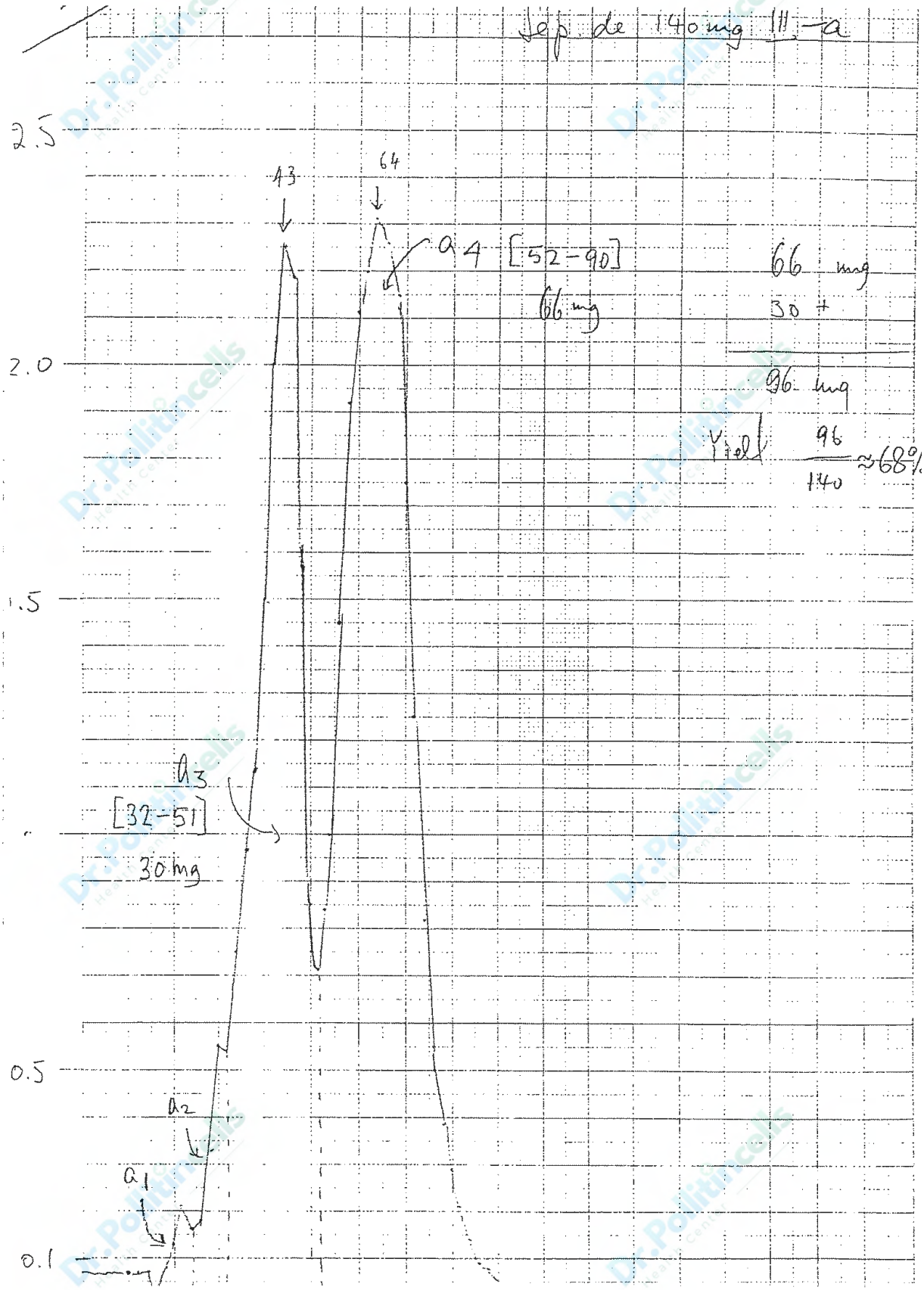
Attn 10 at 280nm

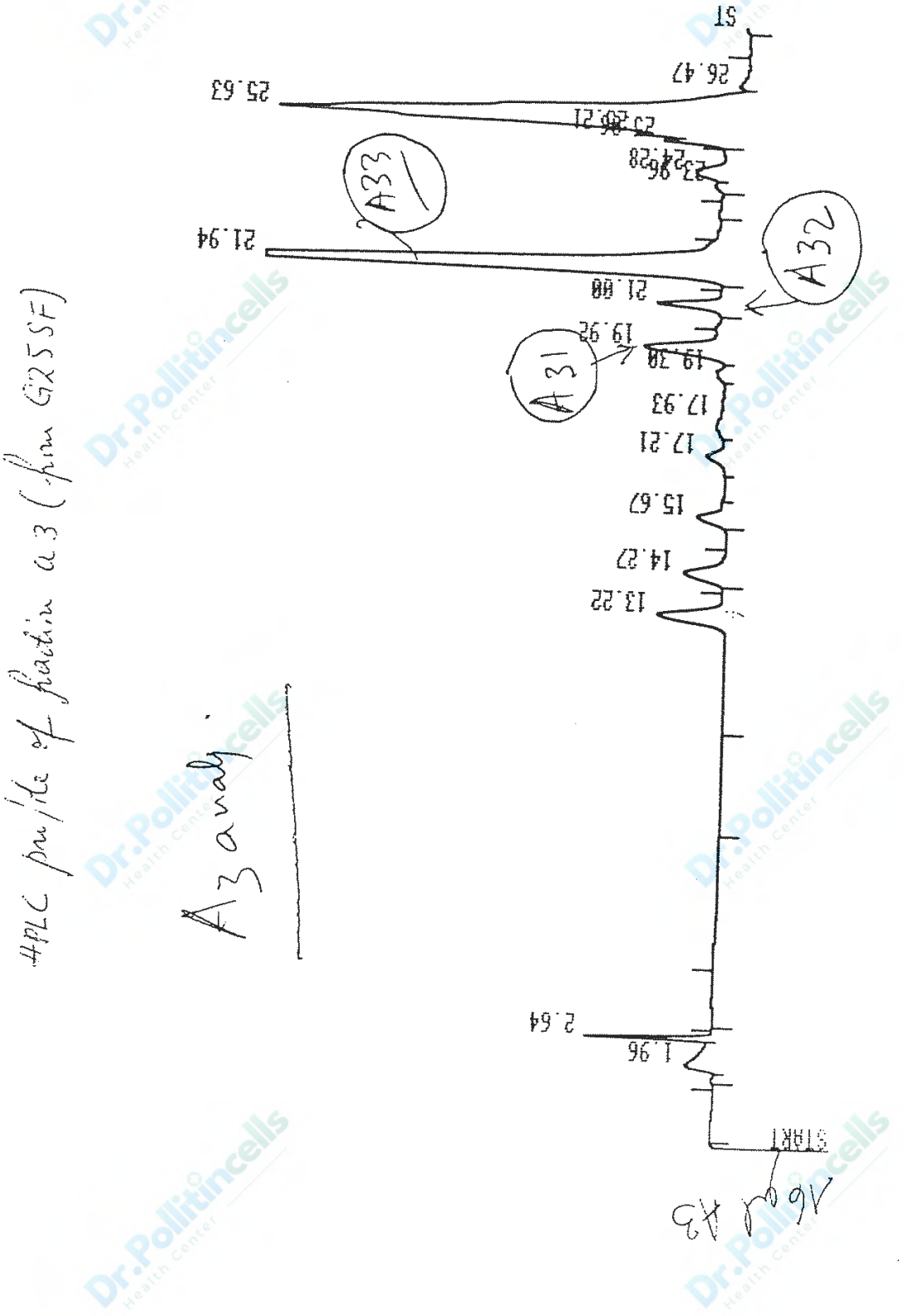


aglucone after hydrolysis at 100°C in 1N HCl

Fig. 8


Run Analytique
5µl
hydrolyzed
b52 (3mg)
under vacuum -
at 295nm.
② 72%
28%
①
ATT 21 6 @
THRSH 5 @
START
1.47
4.67
13.55
13.99
14.78
15.65
16.16
16.52
17.28
17.69
out
20.59
21.53
22.18
b51H
b52H


Sep de 140 mg
2.5
2.0
1.5
0.5
0.1
43
64
a4 [52-90]
66 mg
66 mg
30 +
96 mg
Yield
96/140 ≈ 68%
a3
[32-51]
30 mg
a2
a1


HPLC purification of fraction a3 (from G25SF)
A3 analysis
ST
26.47
25.63
23.96
23.86
23.28
21.94
A33
A32
21.00
19.92
19.38
A31
17.93
17.21
15.67
14.27
13.22
2.64
1.96
START
Area %

HPLC profile of pure b51 after 2 h. boiling
on the Jasmine HPLC column → 2 forms in equilibrium

b51 (in be2HN) 5µl D_2O

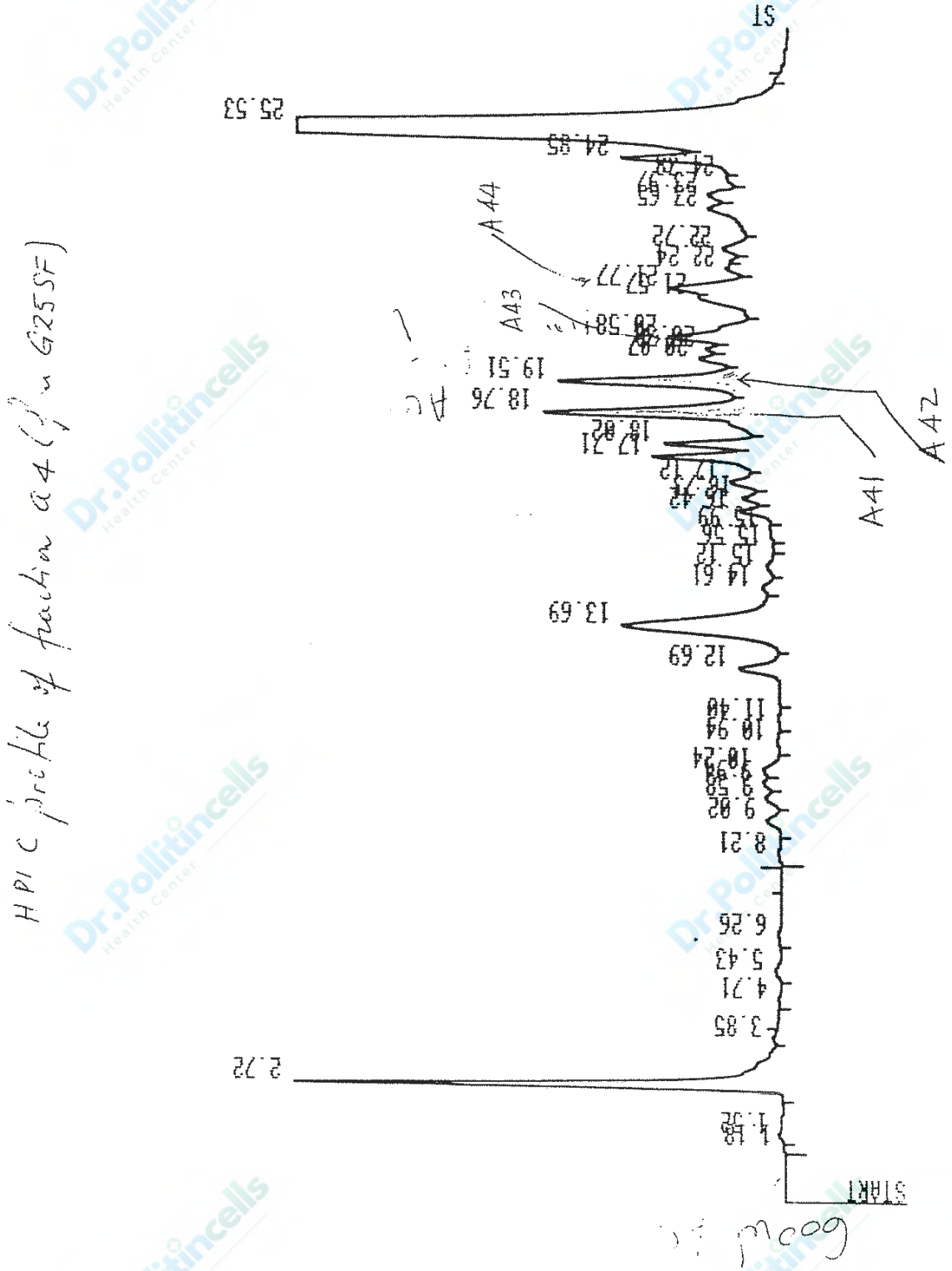
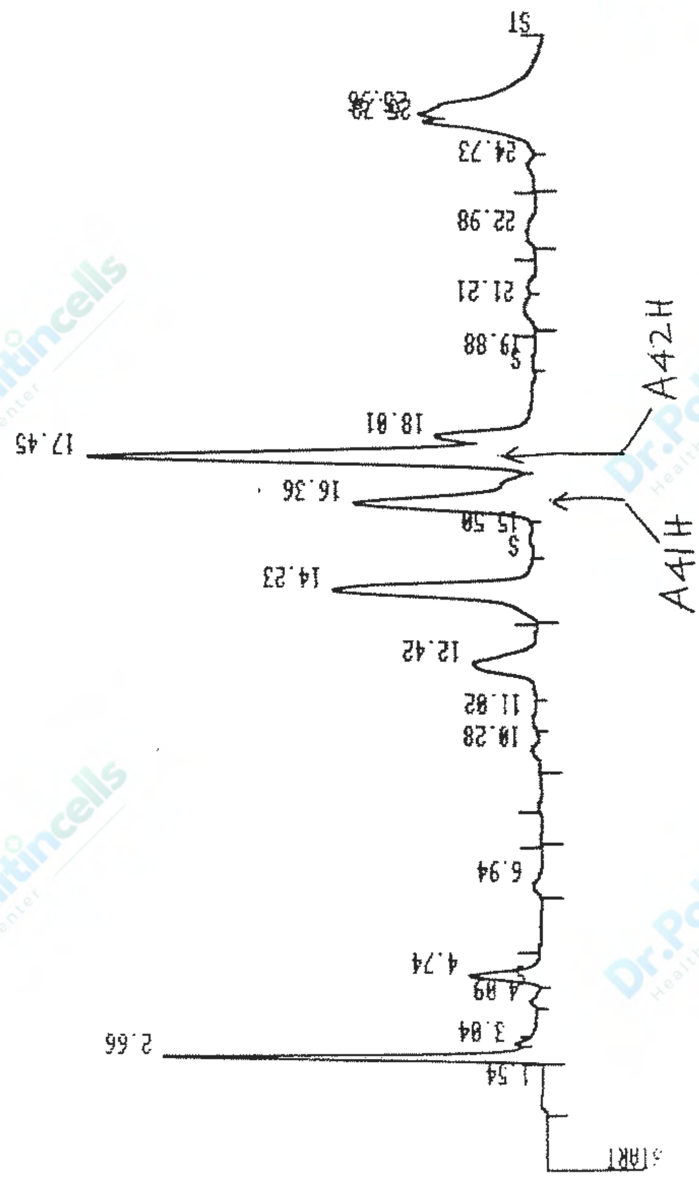

HPLC profile of fraction α4 (γ) on G25SF)
START
1.58
2.72
3.85
4.71
5.43
6.26
8.21
9.02
9.58
10.24
10.94
11.40
12.69
13.69
14.61
15.12
15.56
16.42
17.12
18.21
18.76
19.51
A41
A42
A43
A44
24.85
25.53
ST

Fig 12.

Hydrolysis of a 4 on HPLC



Hydrolysis conditions, as described in the text.