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 I-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

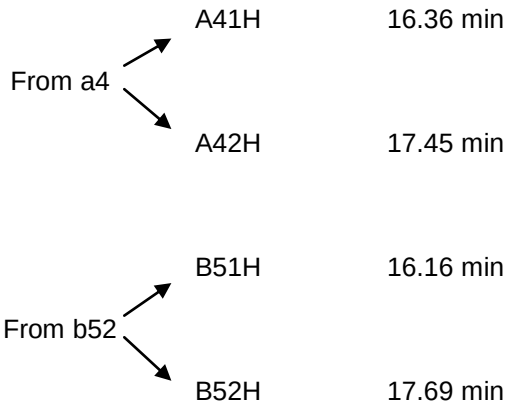
b) the MS spectrum of a41/a42 pair also exhibits degradative material of Mr 1002.2 kDa, i.e. precisely the mass of the pair b51/b52; when one more hexose unit is removed from a41/a42,

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



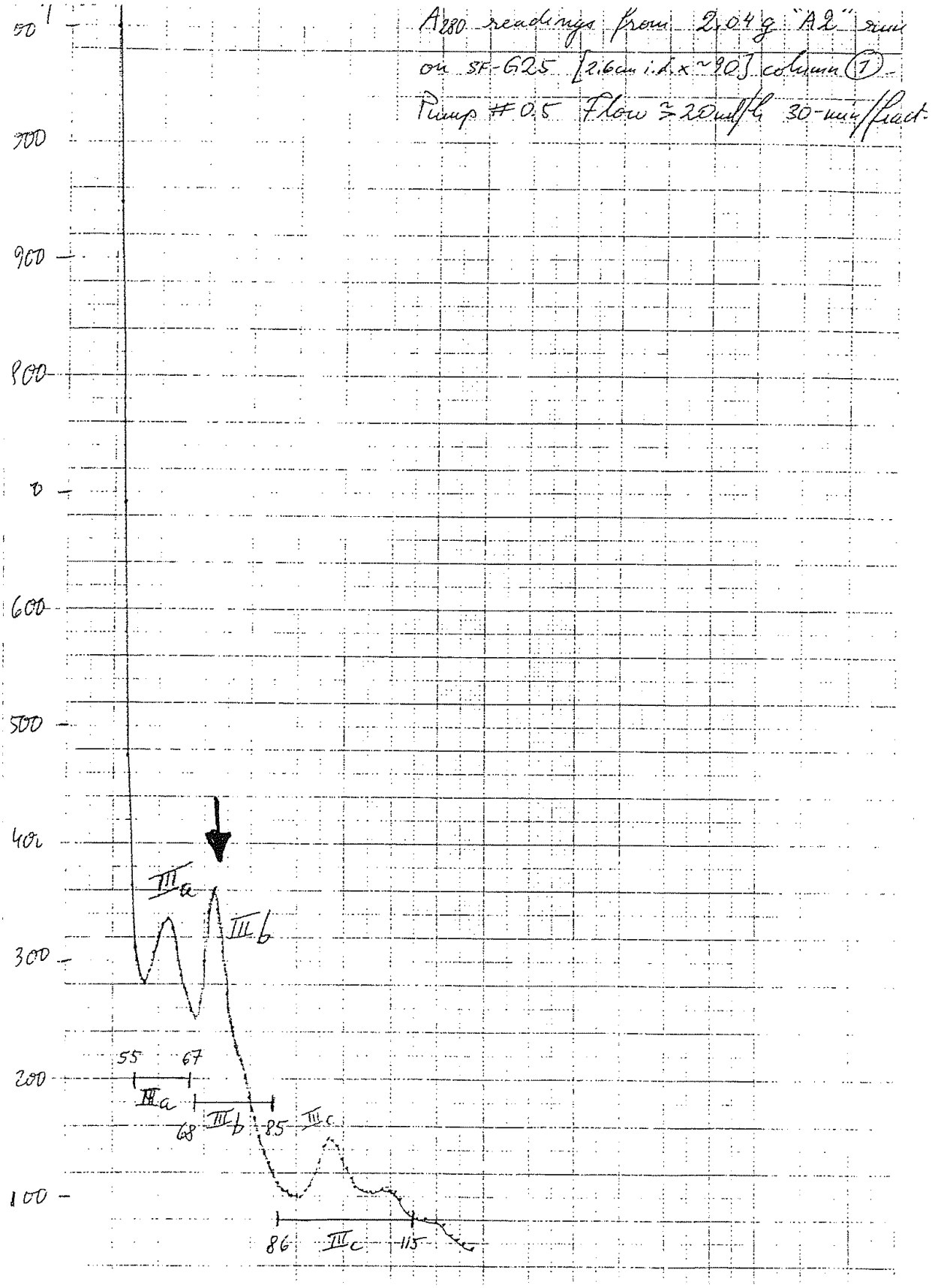
The hydrolyzates from a4 and b52 will be subjected to MS spectroscopy and NMR analyses. The material (~1 mg) was provided on March 29-31, 1993 to Dr. K. Rose (MS) and to Prof. U. Burger (NMR, Sciences II). Very preliminary data from Prof. Burger suggest that that protonic NMR spectra of the hydrolysis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

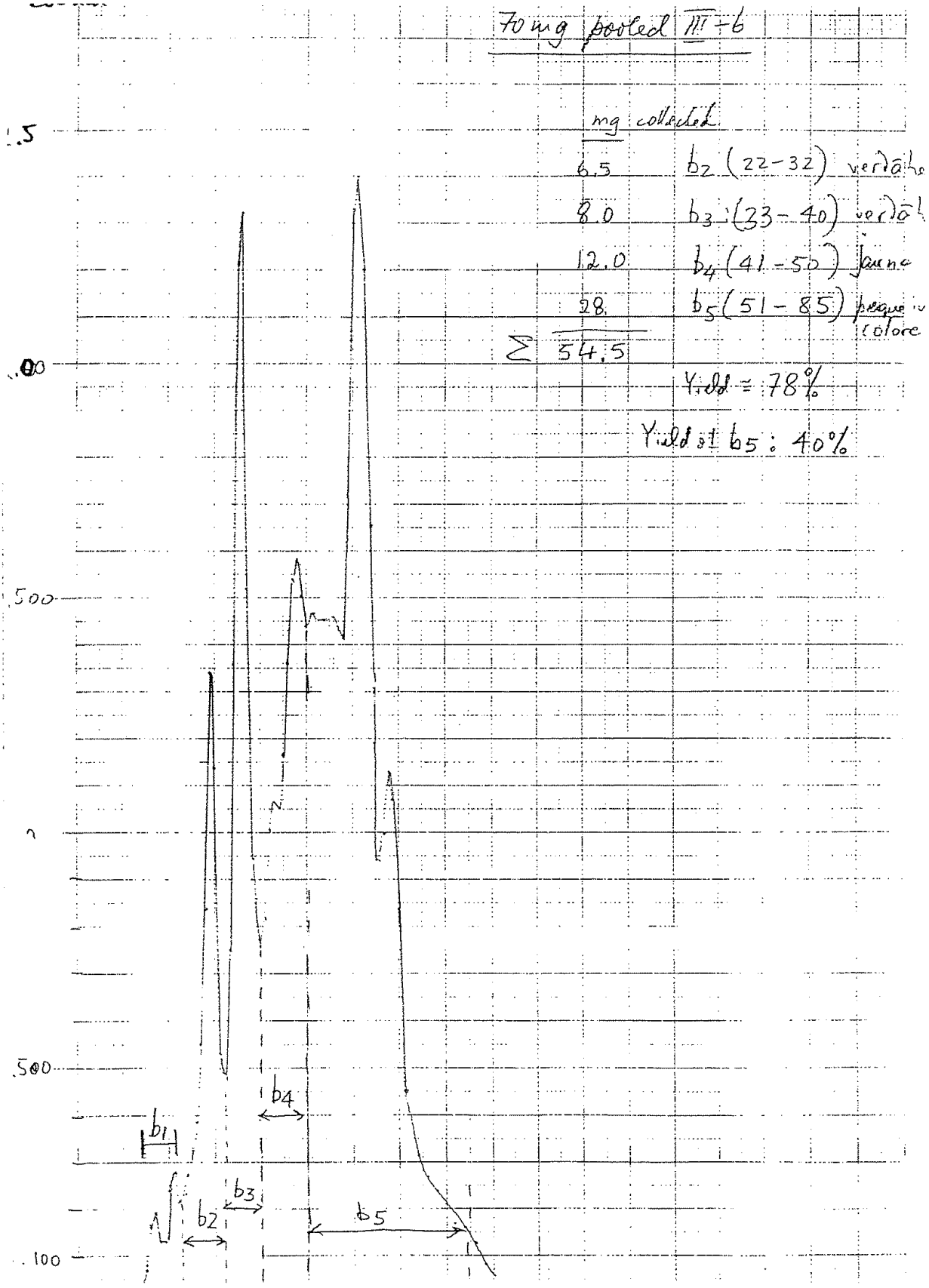
We can, at that time, speculate that biomolecules of the b series (b3, b4 and b5) or of the a series (a3, a4, and a5), which display significant inhibitory activity (Table 1), are all related to each other; they may well differ from each other by the number of hexose units attached to the aglucone moiety.

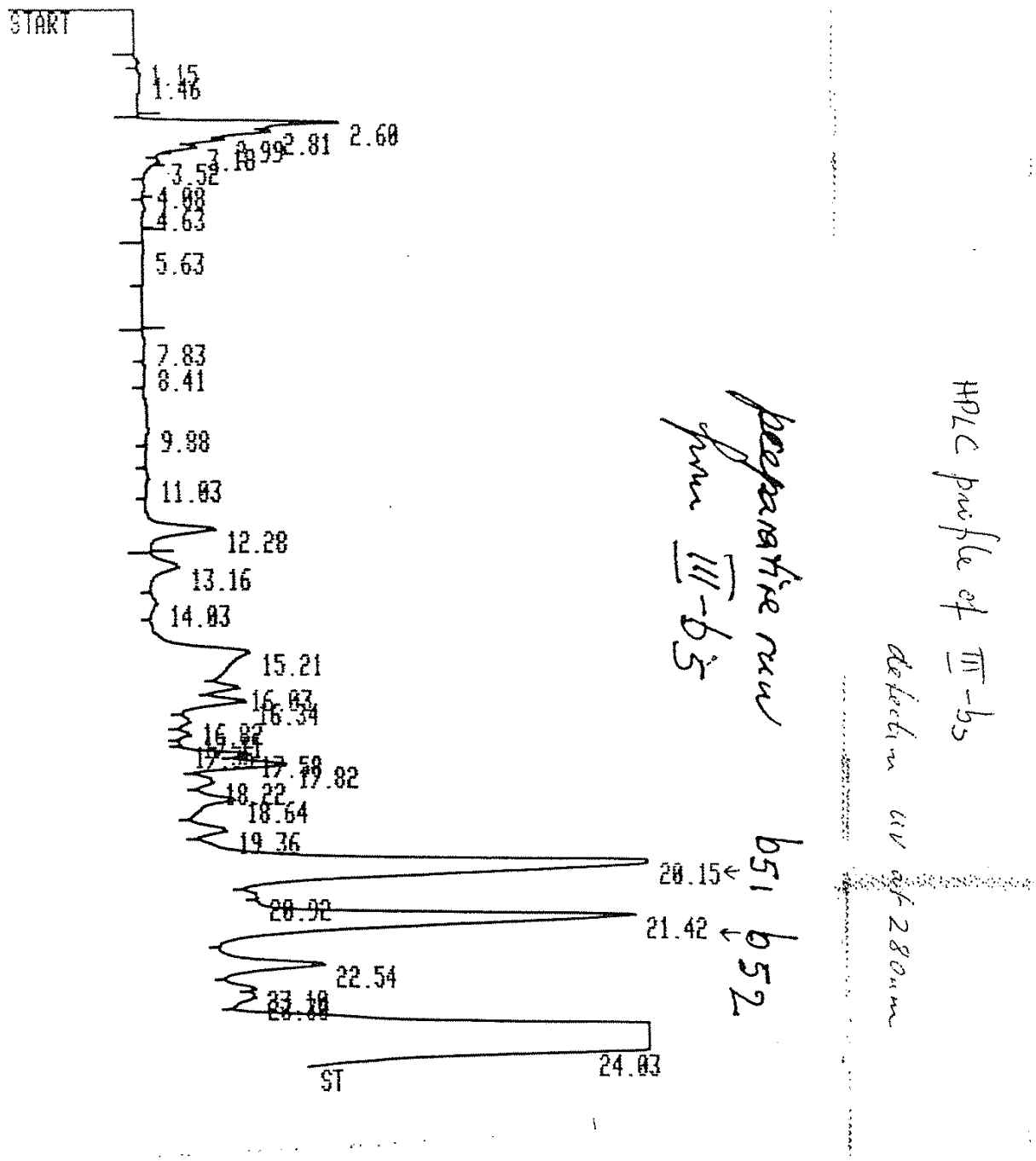
Table 1. *In vitro* bioassay for inhibitory naturally-occurring components from a pollen extract.

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
68
85
III c
86
115


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

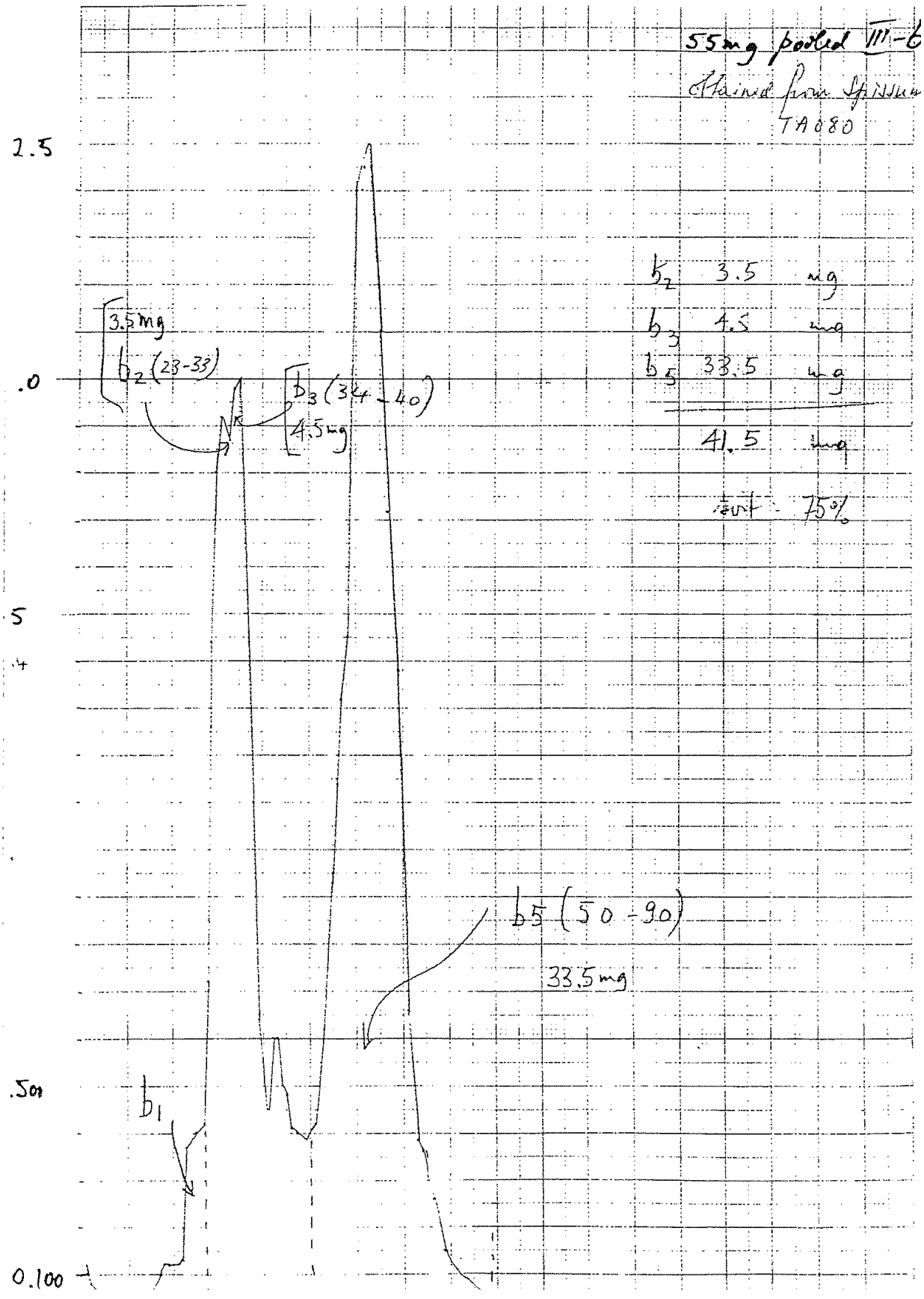


HPLC profile of III-b5 detection uv at 280 nm

preparative run from III-b5

b51 b52

Fig. 3

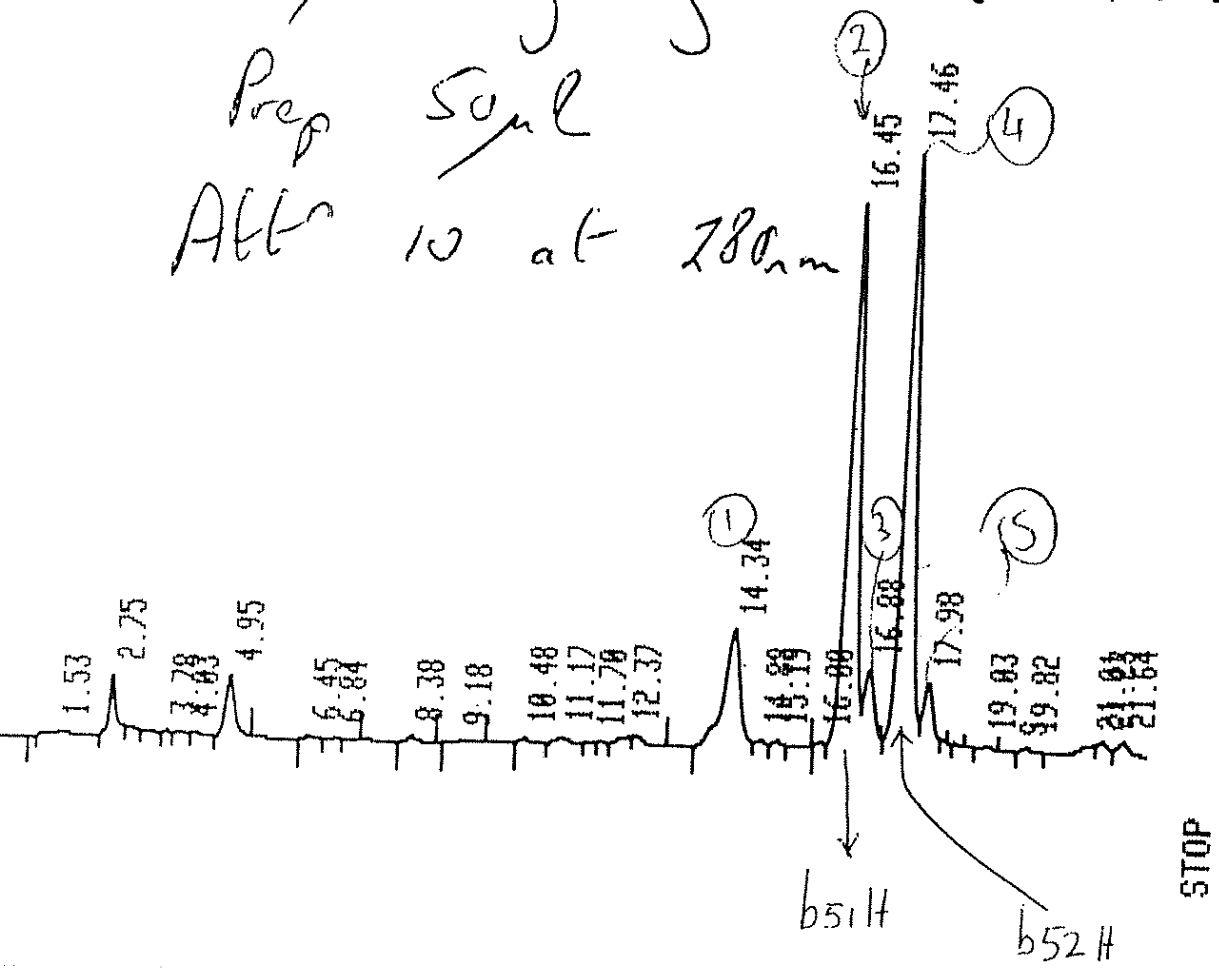

55mg pooled III-6
Obtained from fraction
TA080
2.5
.0
5
.50
0.100
3.5mg
b2 (23-33)
b3 (34-40)
4.5mg
b5 (50-90)
33.5mg
b1
b2 3.5 mg
b3 4.5 mg
b5 33.5 mg
41.5 mg
Fwt - 75%

HILC profile of pure b52 ren. 2 wk later
on the same HILC column → 2 formers in equilibrium
b52 20µl D2O (tube RHN)
START
1.57
3.55
12.62
17.96
17.91
20.45
21.63
23.71
ST

50µl hydrolyzed [redacted] (b51+b52) under no vacuum

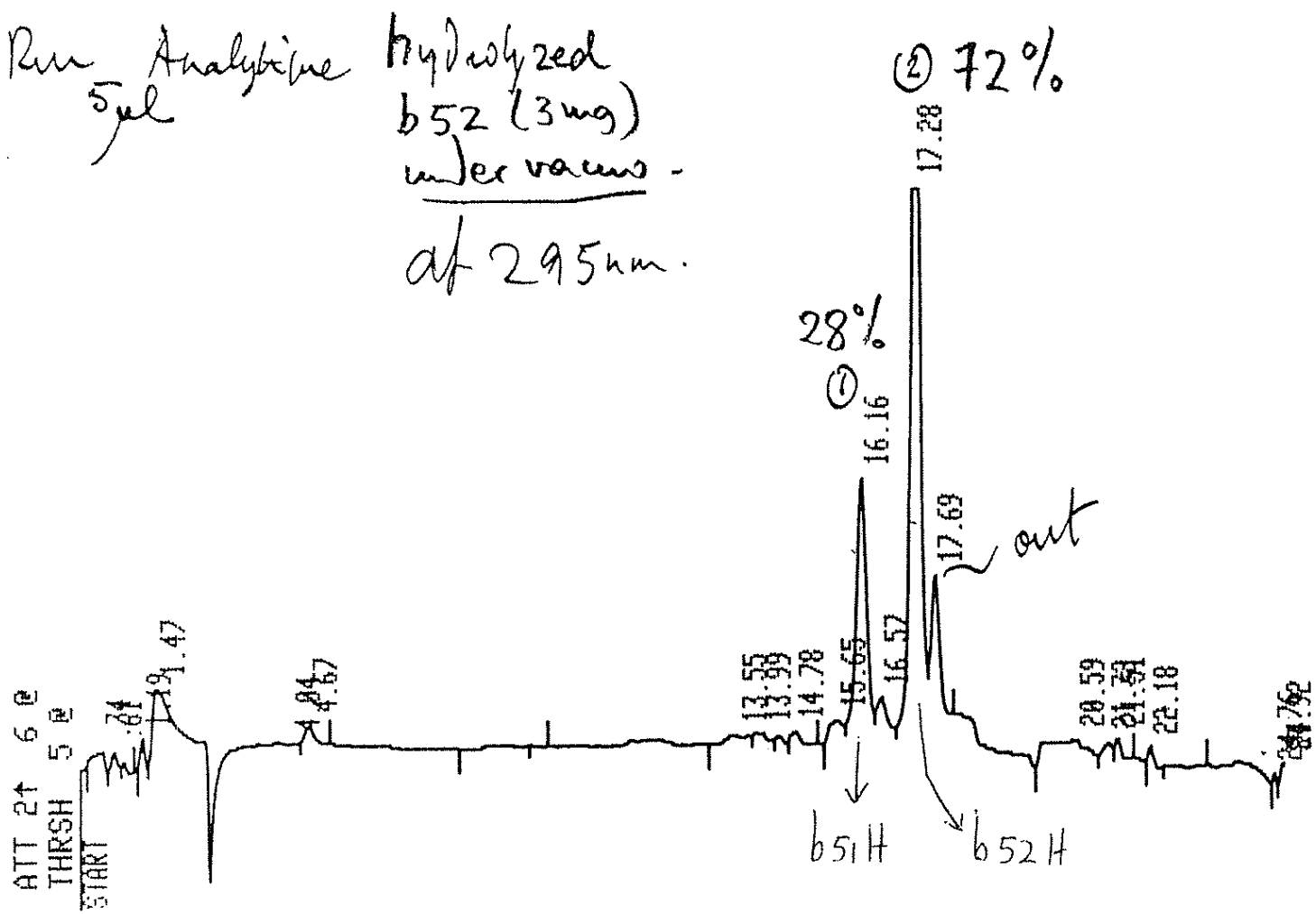
Prep 50µl

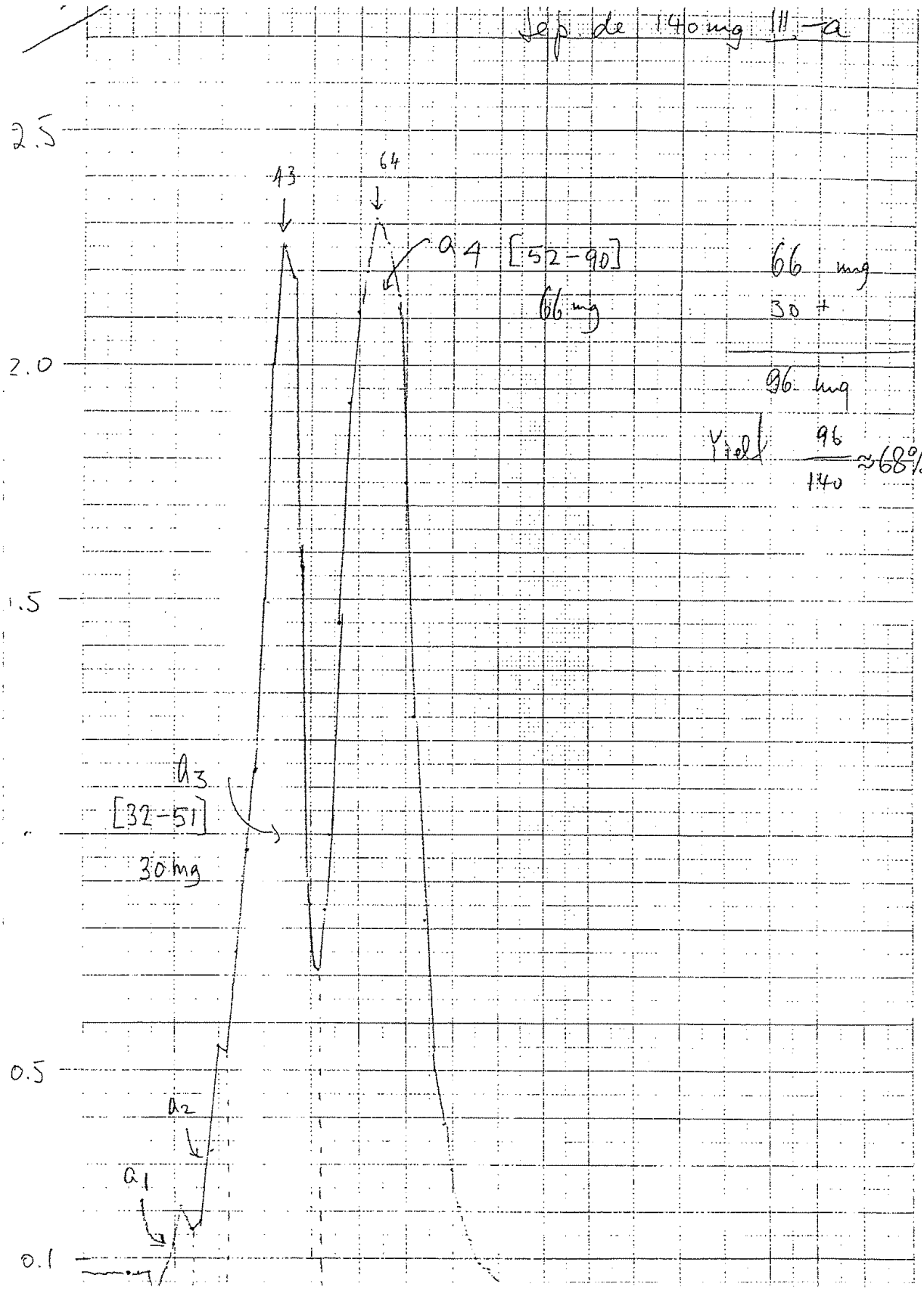
Attn 10 at 280nm

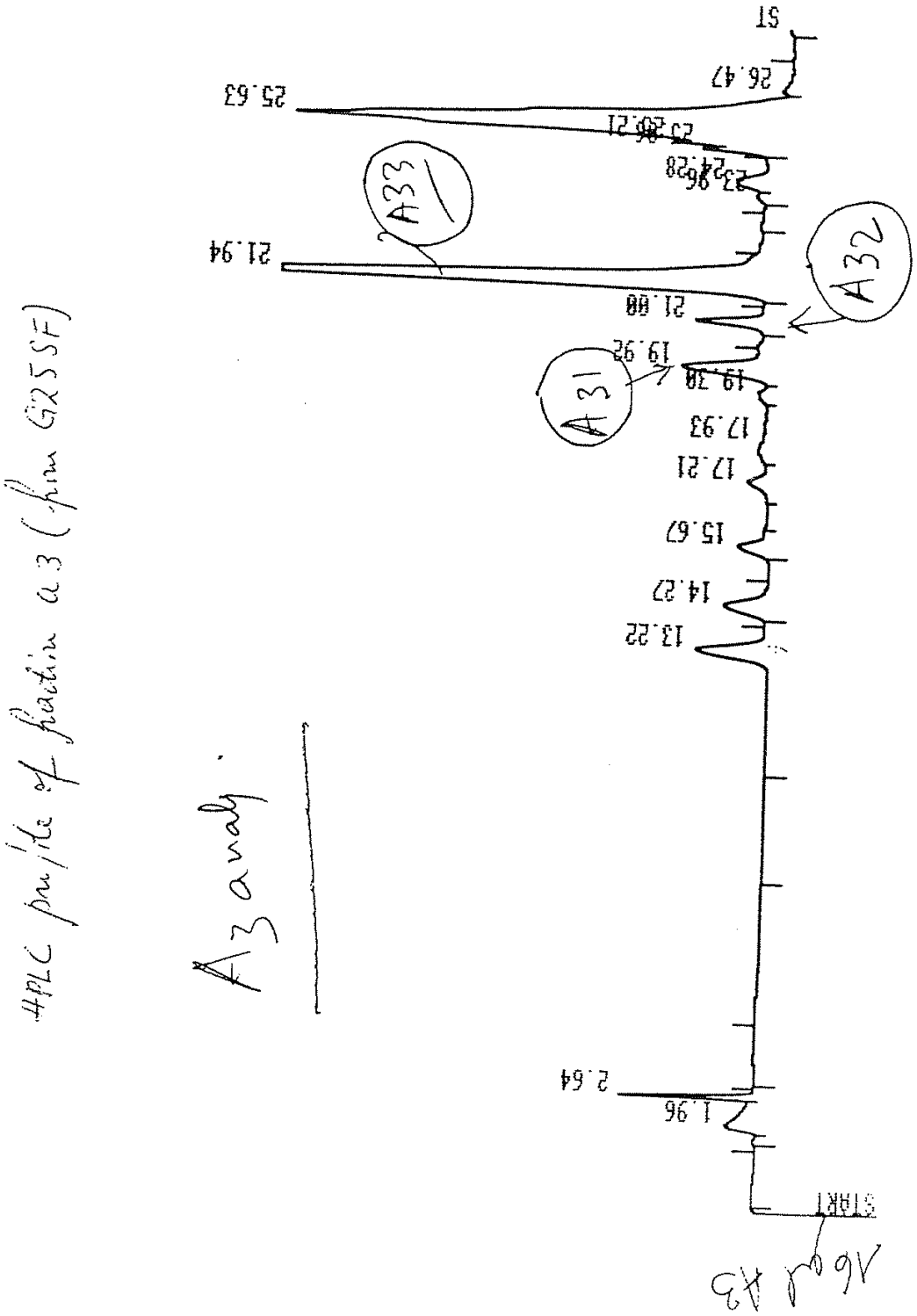


aglucone after hydrolysis at 100°C in 1N HCl

Fig. 8


Run Analytique
5µl
hydrolyzed
b52 (3mg)
under vacuum -
at 295nm.
② 72%
① 28%
ATT 21 6 @
THRSH 5 @
START
1.47
4.67
13.55
13.99
14.78
15.65
16.16
16.57
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 profile of fraction a3 (from G25SF)
A3 analy
Abs 43
START
1.96
2.64
13.22
14.27
15.67
17.21
17.93
19.30
19.92
21.00
21.94
23.96
24.28
25.21
25.63
26.47
ST
A31
A32
A33

HPLC profile of pure b51 verum 2 k. laden
on the Varian HPLC column → 2 forms in equilibrium

b51 (Lubeznin) 5mL D_2O

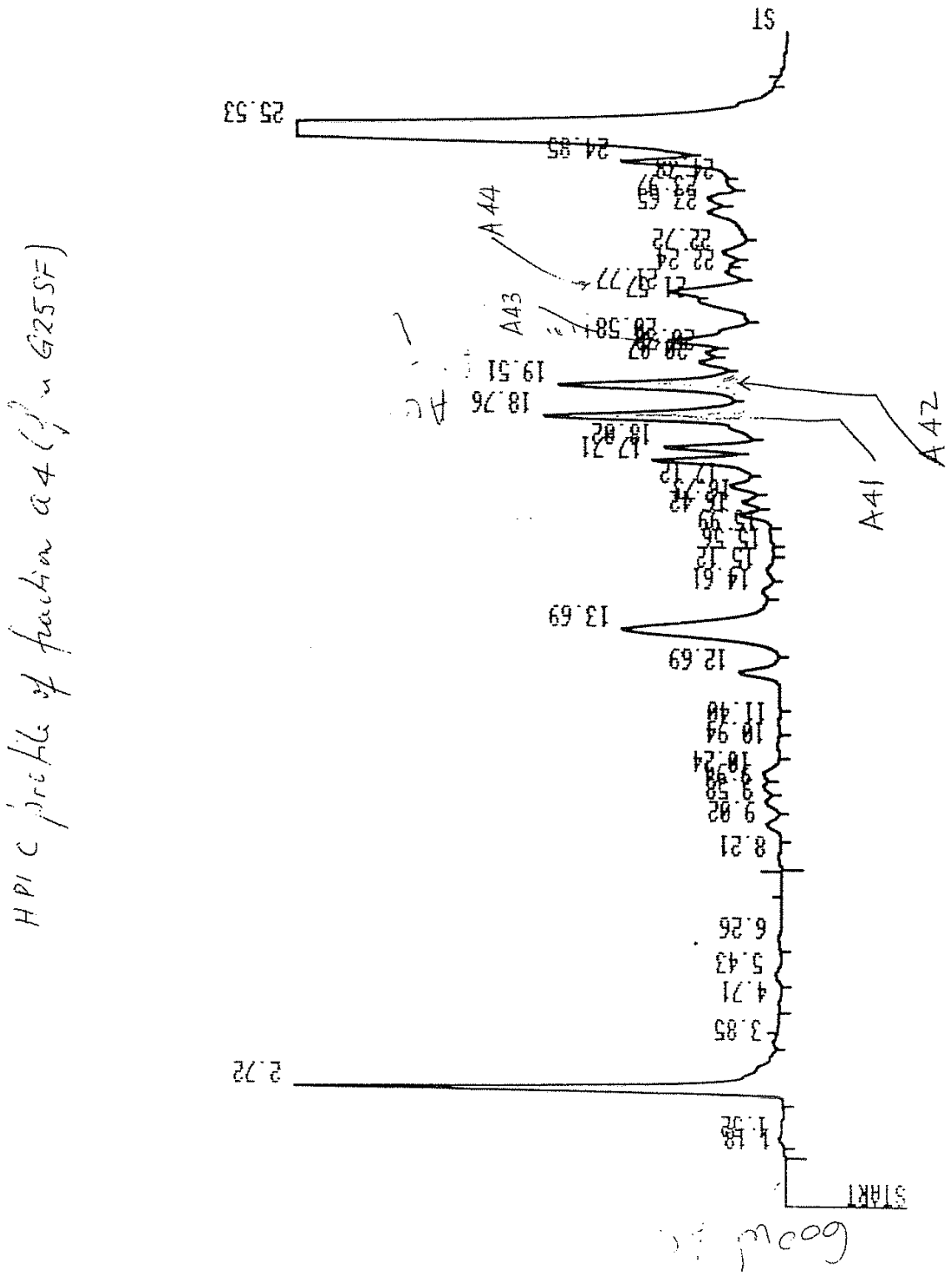
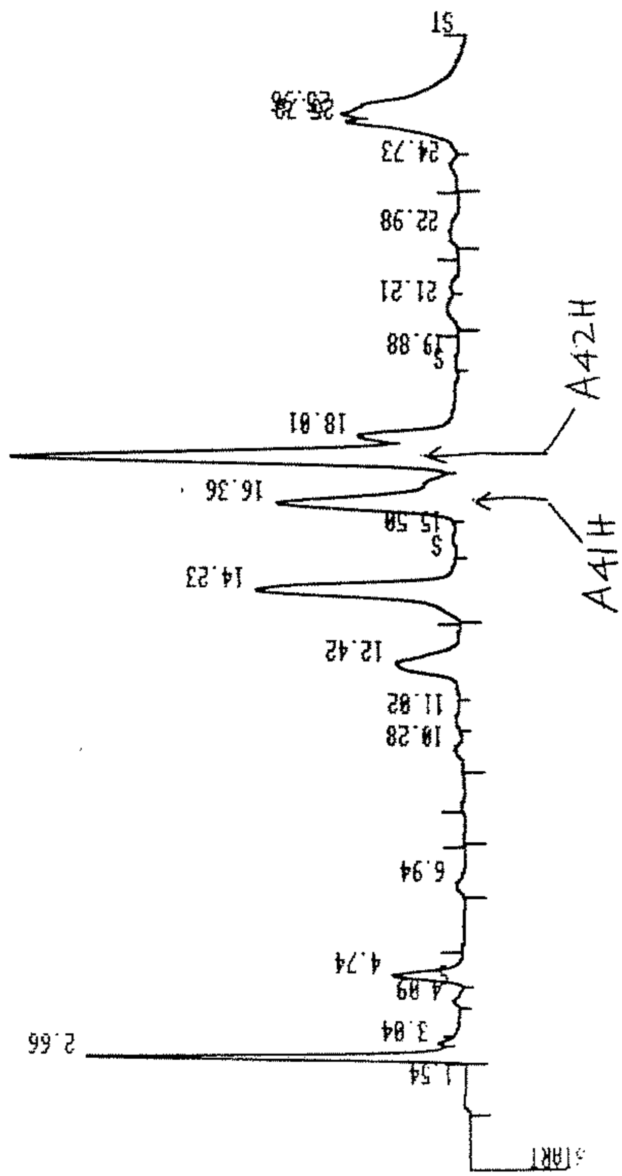

HPLC profile of fraction α4 (γ) on G25SF)
START
2.72
3.85
4.71
5.43
6.26
8.21
9.02
9.58
10.24
10.94
11.40
12.69
13.69
14.61
15.12
15.56
16.42
17.12
18.02
18.76
19.51
A41
A42
A43
A44
24.85
25.53
ST

Fig 12.

Hydrolysis of A4 on HPLC



Hydrolysis conditions as described in the text.