US006316214B1

(12) **United States Patent**
Rinehart et al.

(10) **Patent No.: US 6,316,214 B1**
(45) **Date of Patent: Nov. 13, 2001**

(54) **ETM-775 METABOLITE OF ECTEINASCIDIN 743**

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(73) Assignee: **The Board of Trustees of the University of Illinois**, Champaign, IL (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/309,947**

(22) Filed: **May 11, 1999**

Related U.S. Application Data

(60) Provisional application No. 60/085,024, filed on May 11, 1998.

(51) **Int. Cl.**[7] **C12Q 1/26**
(52) **U.S. Cl.** **435/25**; 514/250; 544/233
(58) **Field of Search** 435/25; 514/250; 544/233

(56) **References Cited**

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4,273,773		6/1981	Demerson et al.	424/250
5,089,273		2/1992	Rinehart et al.	424/520
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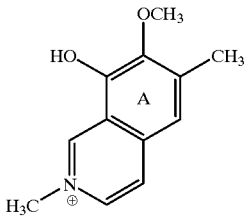
* cited by examiner

Primary Examiner—Ralph Gitomer
(74) *Attorney, Agent, or Firm*—Ernest V. Linek; Banner & Witcoff, Ltd.

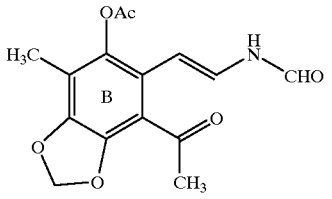
(57) **ABSTRACT**

The purification and structure elucidation of several products of the metabolism of Et 743 by human cytochrome CYP3A4 have been accomplished. These compounds are abbreviated herein as "ETM" followed by a numeric value, which represents the approximate molecular weight. Three compounds have been identified to date, namely ETM 305, ETM 775 and ETM 204. The structures of these ecteinascidin metabolites are as follows:

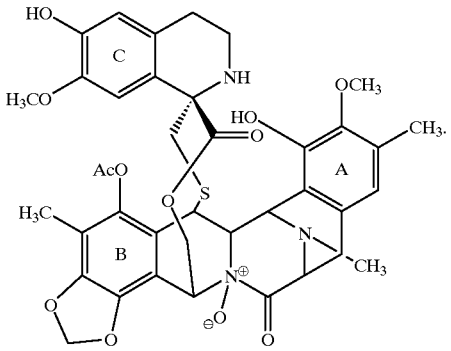
ETM 204



ETM 305



ETM 775



2 Claims, 25 Drawing Sheets

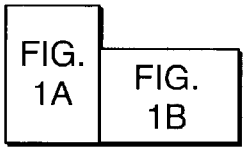


FIG. 1

MORALES, KLR, ETH-SIOH-1 IN CDC13

EXPL S3PUL

SAMPLE	
DATE	FEB 27 98
SOLVENT	CDC 13
FILE	EXP
ACQUISITION	
STFRQ	499.699
TN	111
AT	3.277
NP	39296
SW	5996.1
FB	3400
BS	16
TPWR	63
PW	4.7
DL	0
TOF	0
NL	400
CT	160
ALOCK	N
GAIN	NOT USED
FLAGS	
11	N
LN	N
DP	Y
HS	NN
DISPLAY	
SP	-138.2
WP	5133.1
V$	8848
SC	0
WC	250
NIMM	20.53
LS	33.57
RFL	4131.0
RFP	3627.8
TH	7
INS	1.000
NM	PH

DEC. & VT	
DFRQ	499.699
DN	HL
DPWR	20
DOF	6
DM	NNN
DMM	C
DMF	200
DSEQ	
DRES	1.0
HOMO	N
DEC2	
DFRQ2	0
DN2	
DPWR2	1
DOF2	0
DM2	N
DMM2	C
DMF2	200
DSEQ2	
DRES2	1.0
HOMO2	N
PROCESSING	
16	6.30
WTFILE	
PROC	FT
FN	NOT USED
MATH	R
WERR	
WEXP	
WBS	
WNT	

FIG. 1A

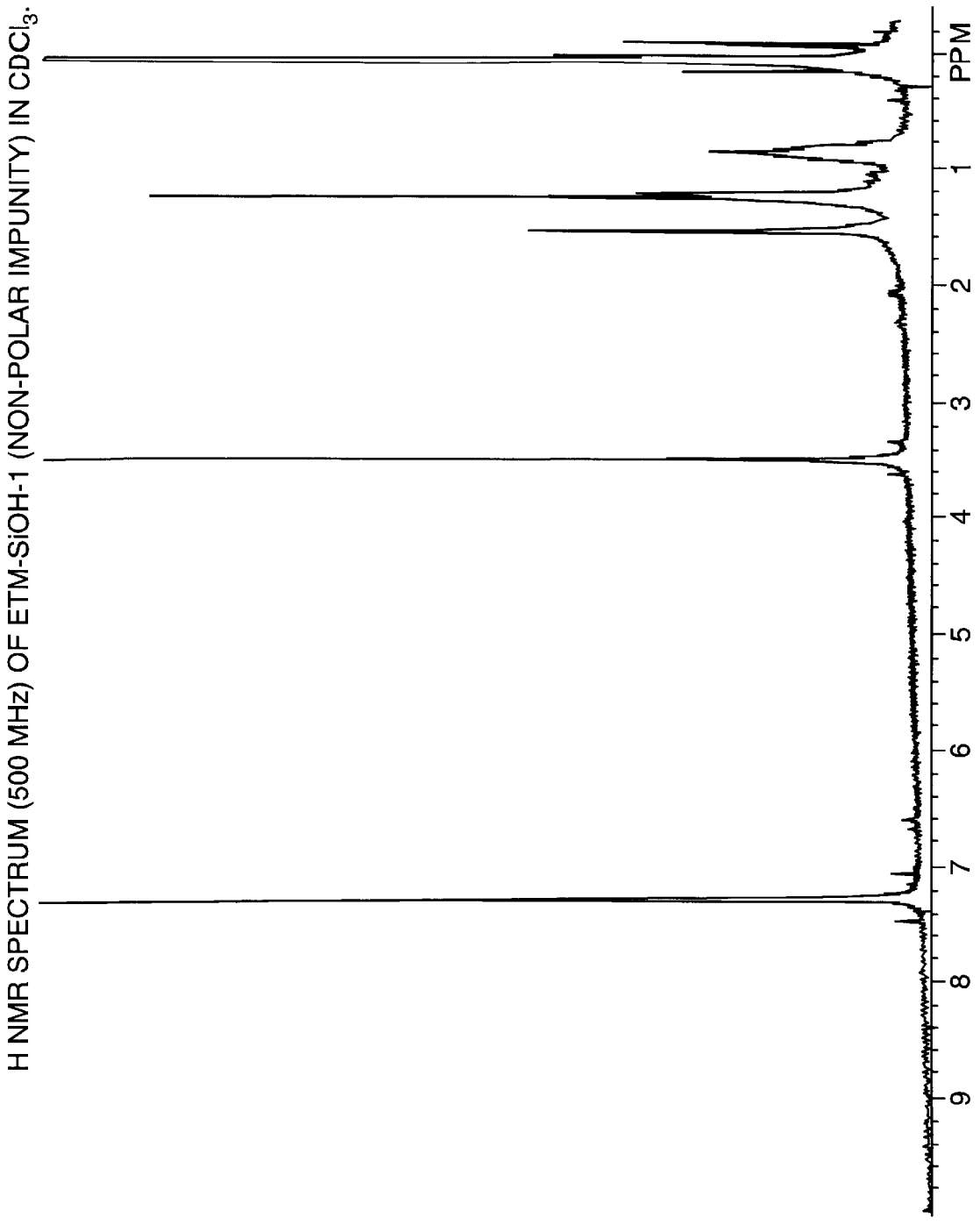
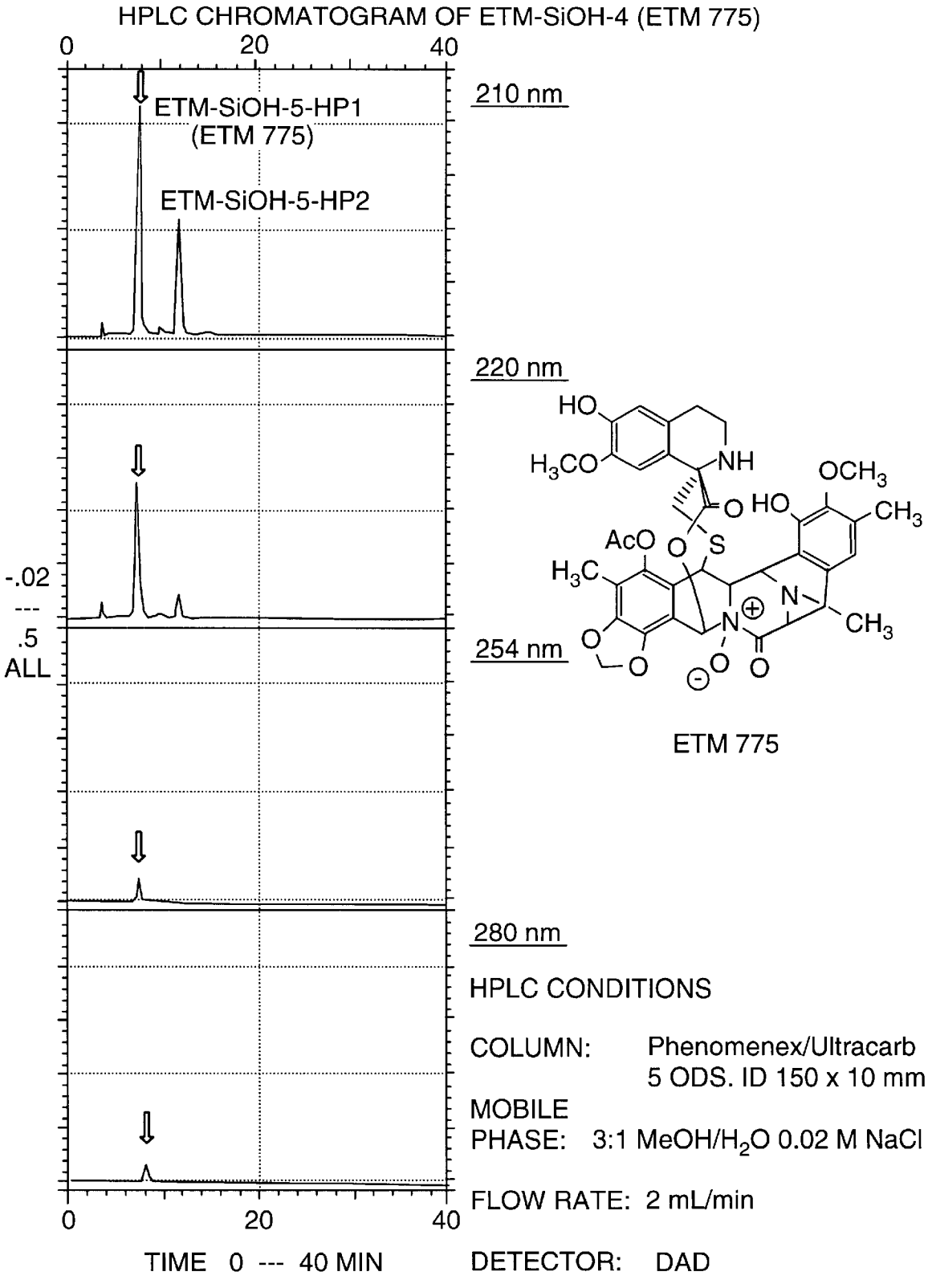

H NMR SPECTRUM (500 MHz) OF ETM-SiOH-1 (NON-POLAR IMPUNITY) IN $CDCl_3$.
PPM
9
8
7
6
5
4
3
2
1

FIG. 1B


HPLC CHROMATOGRAM OF ETM-SiOH-4 (ETM 775)
0
20
40
ETM-SiOH-5-HP1
(ETM 775)
ETM-SiOH-5-HP2
210 nm
220 nm
254 nm
280 nm
-.02

.5
ALL
HO
H3CO
NH
OCH3
HO
CH3
AcO
O
S
H3C
N
N
CH3
O
O
O
O
O
ETM 775
HPLC CONDITIONS
COLUMN: Phenomenex/Ultracarb
5 ODS. ID 150 x 10 mm
MOBILE
PHASE: 3:1 MeOH/H2O 0.02 M NaCl
FLOW RATE: 2 mL/min
DETECTOR: DAD
0
20
40
TIME 0 --- 40 MIN

FIG. 2

HPLC CHROMATOGRAM OF ETM-SiOH-3 (ETM 305)

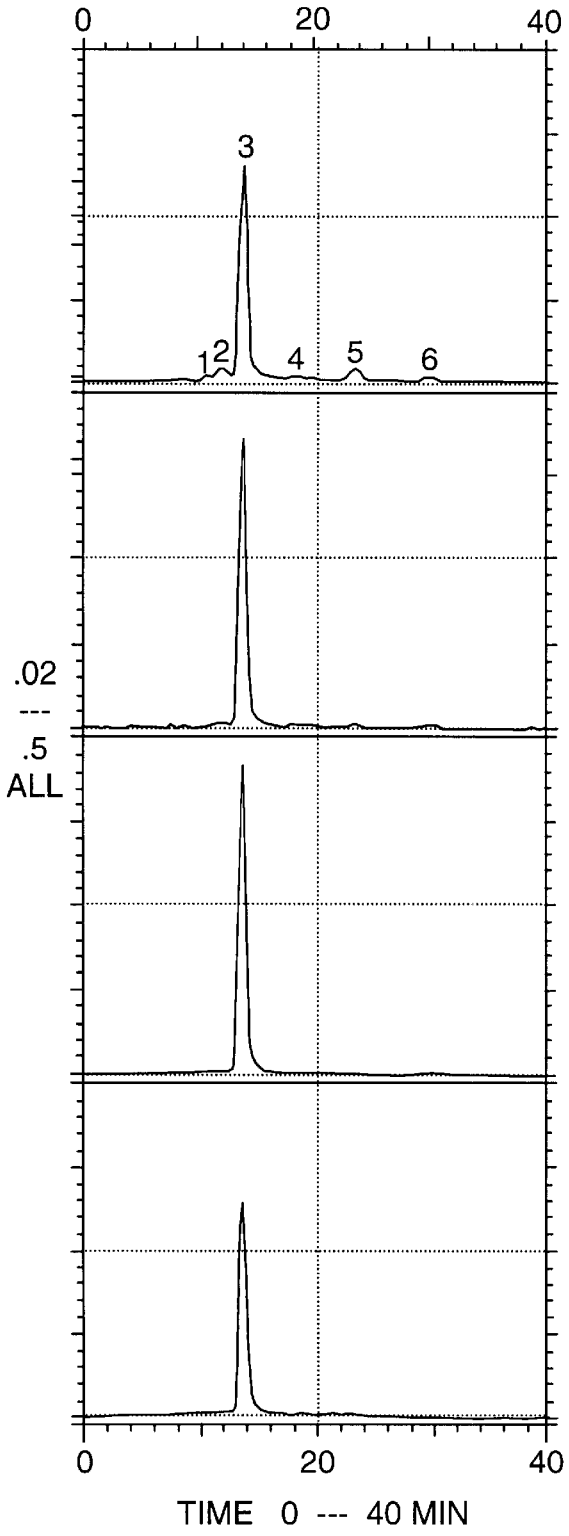
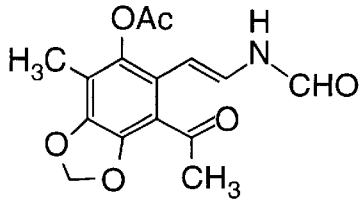


FIG. 3



ETM 305

HPLC CONDITIONS

COLUMN: Phenomenex/Ultracarb 5 ODS. ID 150 x 10 mm

MOBILE PHASE: 3:1 MeOH/H_2O 0.02 M NaCl

FLOW RATE: 1mL/min

DETECTOR: DAD

HPLC CHROMATOGRAM OF ETM-SiOH-2 (TRACE METABOLITES).

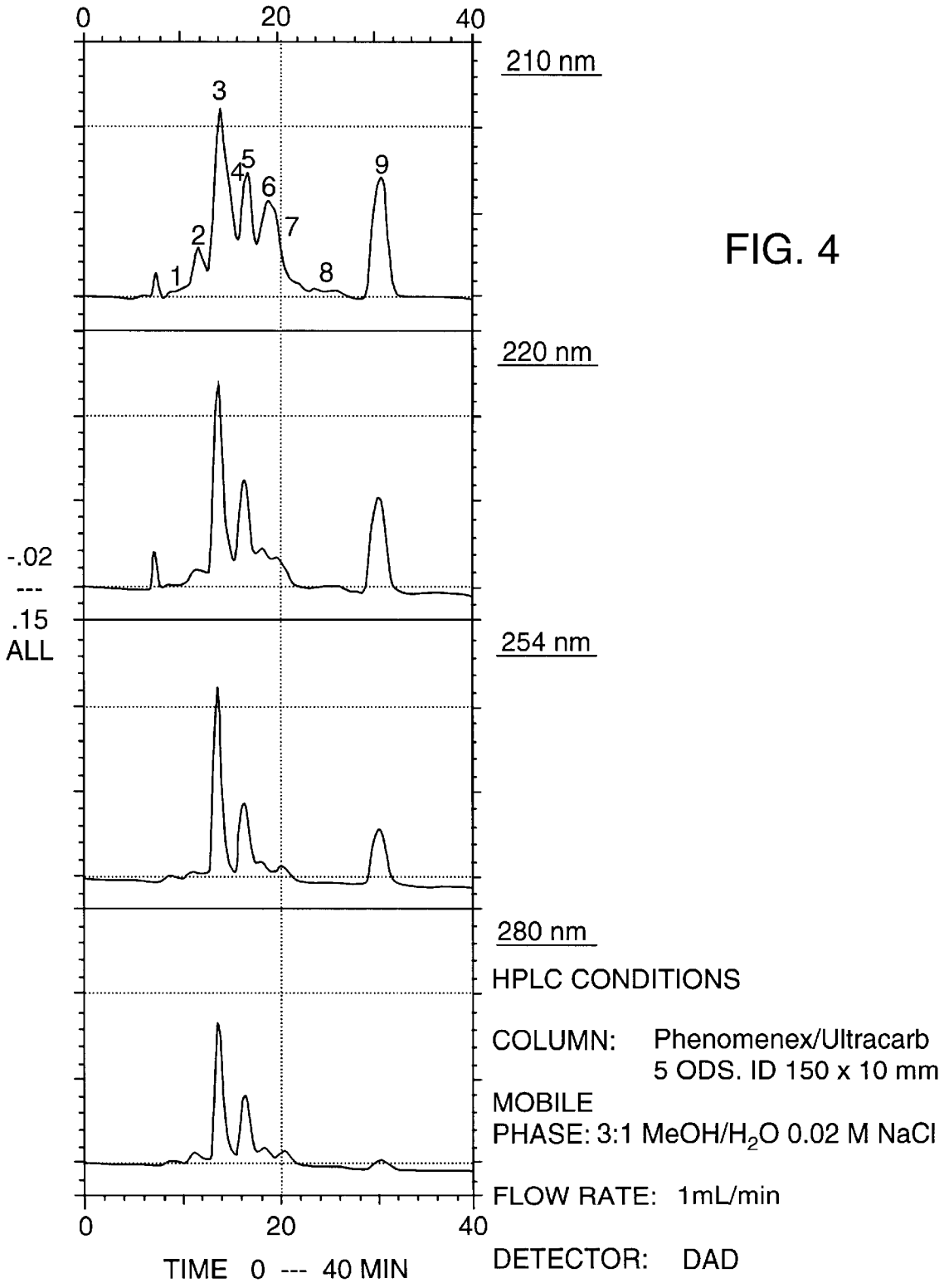


FIG. 4

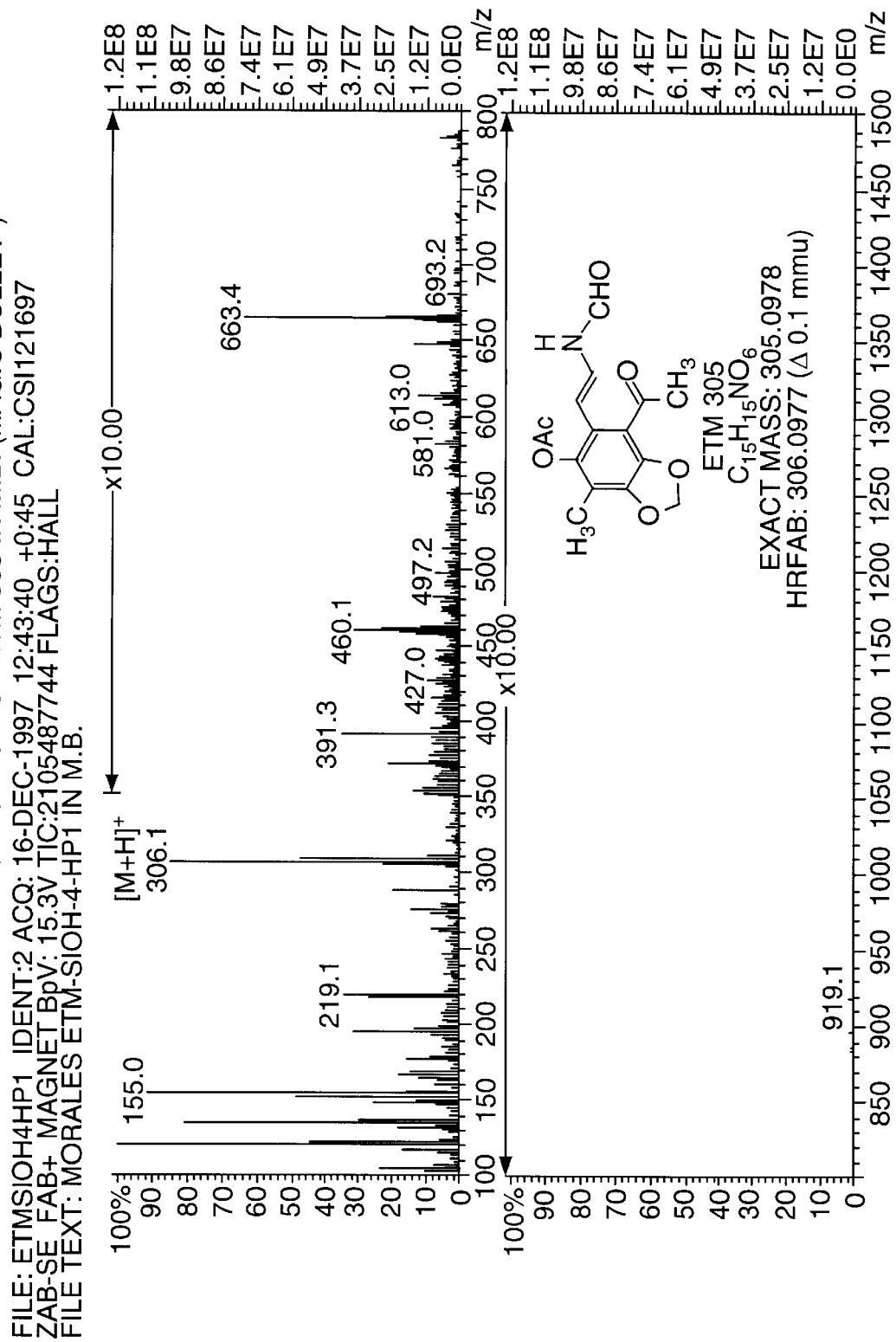

LRFAB MASS SPECTRUM OF ETM 305 IN M.B. (MAGIC BULLET4)
FILE: ETMSIOH4HP1 IDENT:2 ACQ: 16-DEC-1997 12:43:40 +0:45 CAL:CSI121697
ZAB-SE FAB+ MAGNET BpV: 15.3V TIC:2105487744 FLAGS:HALL
FILE TEXT: MORALES ETM-SIOH-4-HP1 IN M.B.
[M+H]+
306.1
155.0
219.1
391.3
460.1
427.0
497.2
581.0
613.0
663.4
693.2
919.1
x10.00
m/z
OAc
H
N
CHO
H3C
O
CH3
ETM 305
C15H15NO6
EXACT MASS: 305.0978
HRFAB: 306.0977 (Δ 0.1 mmu)

FIG. 5

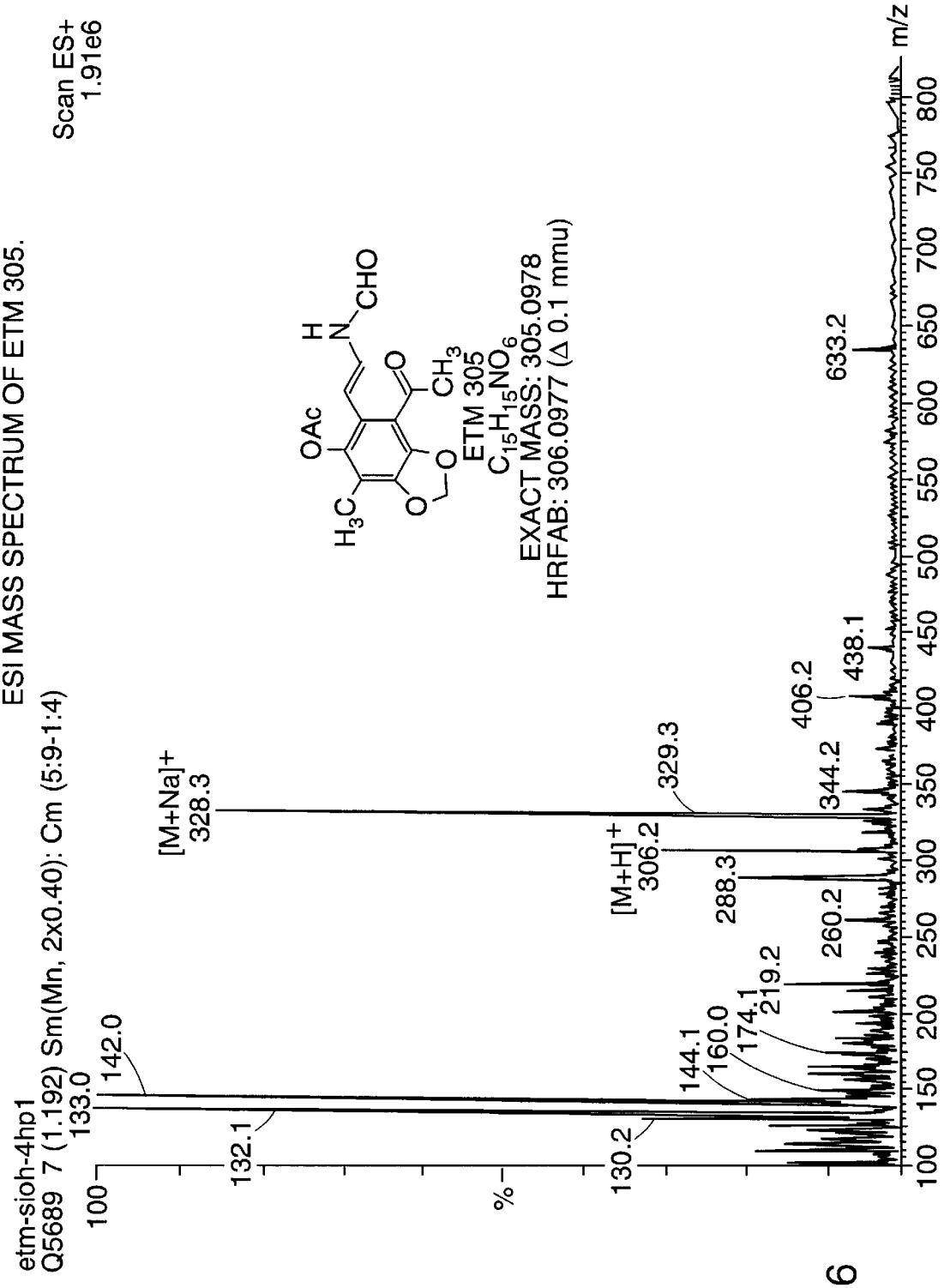

ESI MASS SPECTRUM OF ETM 305.
etm-sioh-4hp1
Q5689 7 (1.192) Sm(Mn, 2x0.40): Cm (5:9-1:4)
Scan ES+
1.91e6
OAc
H
N
CHO
H_3C
O
CH_3
O
O
ETM 305
$C_{15}H_{15}NO_6$
EXACT MASS: 305.0978
HRFAB: 306.0977 (Δ 0.1 mmu)
[M+Na]+
328.3
[M+H]+
306.2
329.3
406.2
438.1
344.2
633.2
288.3
260.2
219.2
174.1
160.0
144.1
142.0
133.0
132.1
130.2
100
%
m/z
100
150
200
250
300
350
400
450
500
550
600
650
700
750
800

FIG. 6

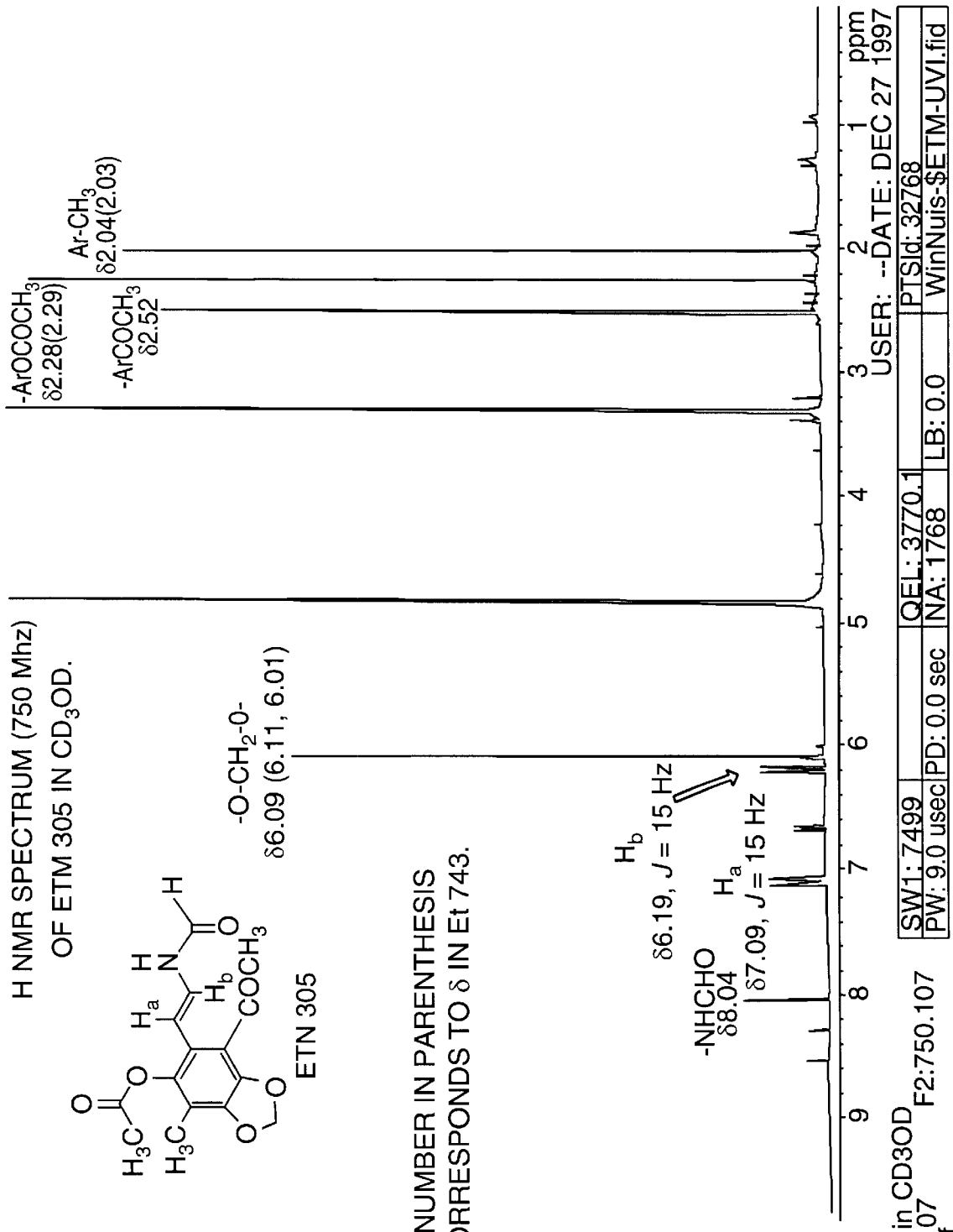

H NMR SPECTRUM (750 Mhz)
OF ETM 305 IN CD_3OD.
ETN 305
*NUMBER IN PARENTHESIS
CORRESPONDS TO δ IN Et 743.
-ArOCOCH3 δ2.28(2.29)
-ArCOCH3 δ2.52
Ar-CH3 δ2.04(2.03)
-O-CH2-O- δ6.09 (6.11, 6.01)
Hb δ6.19, J = 15 Hz
-NHCHO δ8.04
Ha δ7.09, J = 15 Hz
1-UV in CD3OD
750.107
s2puf
F2:750.107
SW1: 7499
PW: 9.0 usec
PD: 0.0 sec
QEL: 3770.1
NA: 1768
LB: 0.0
PTSId: 32768
WinNuis-$ETM-UVI.fid
USER: --DATE: DEC 27 1997
ppm

FIG. 7

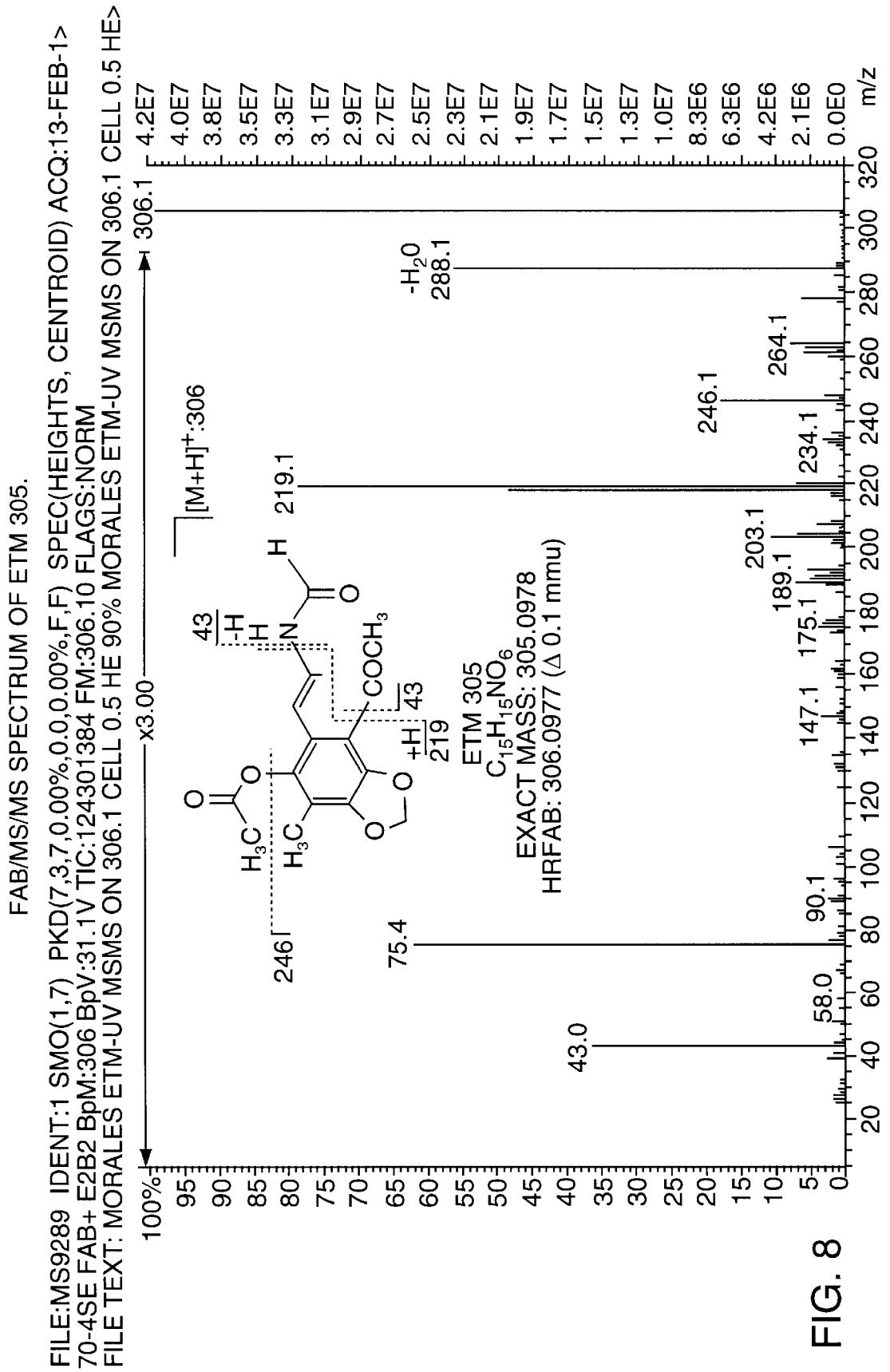

FAB/MS/MS SPECTRUM OF ETM 305.
FILE:MS9289 IDENT:1 SMO(1,7) PKD(7,3,7,0.00%,0.0,0.00%,F,F) SPEC(HEIGHTS, CENTROID) ACQ:13-FEB-1>
70-4SE FAB+ E2B2 BpM:306 BpV:31.1V TIC:124301384 FM:306.10 FLAGS:NORM
FILE TEXT: MORALES ETM-UV MSMS ON 306.1 CELL 0.5 HE 90% MORALES ETM-UV MSMS ON 306.1 CELL 0.5 HE>
100%
x3.00
306.1
[M+H]+:306
219.1
-H2O
288.1
246.1
264.1
234.1
203.1
189.1
175.1
147.1
90.1
75.4
58.0
43.0
246
219
43
-H
+H
COCH3
H3C
H3C
O
O
O
O
N
H
ETM 305
C15H15NO6
EXACT MASS: 305.0978
HRFAB: 306.0977 (Δ 0.1 mmu)
m/z

FIG. 8

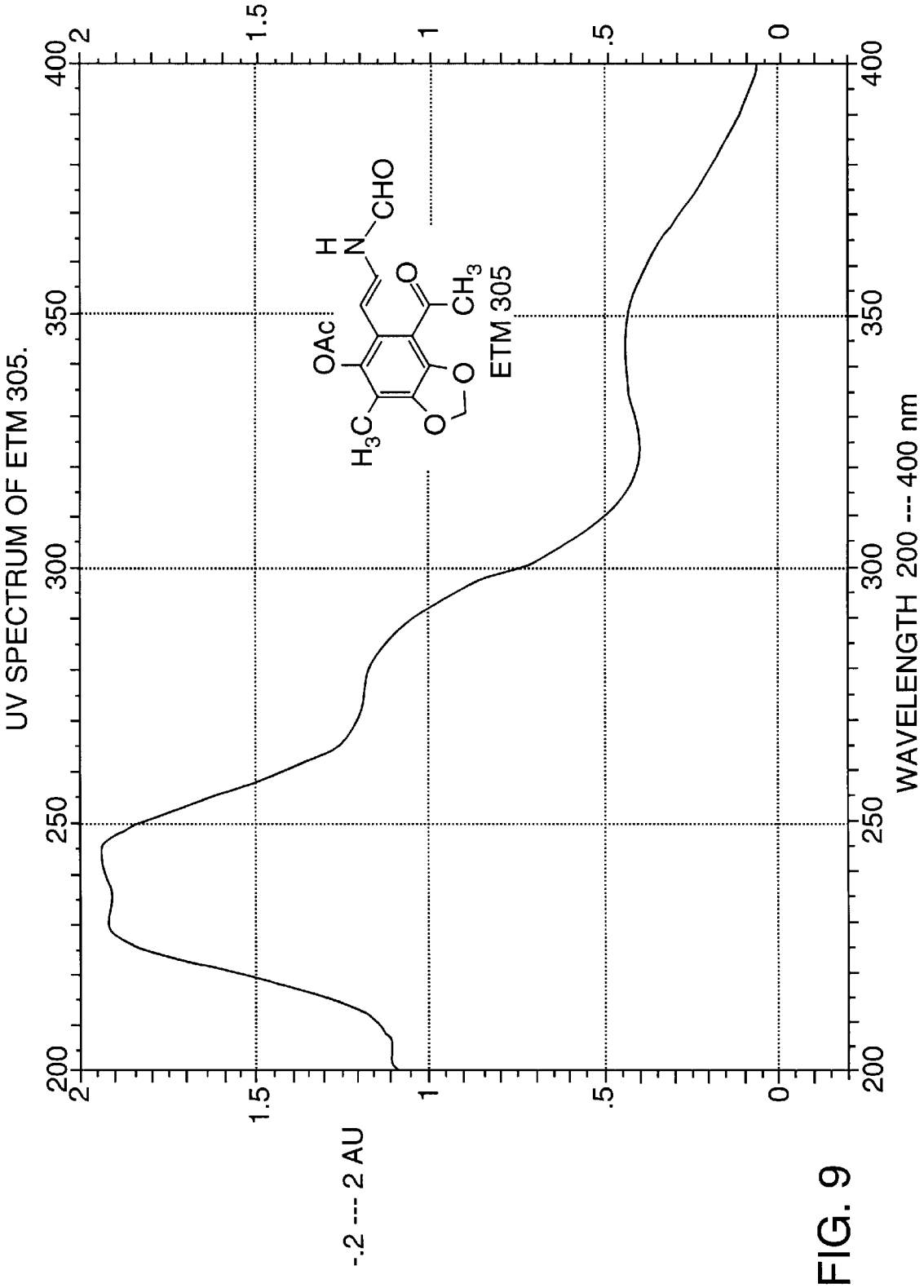

UV SPECTRUM OF ETM 305.
-.2 --- 2 AU
WAVELENGTH 200 --- 400 nm
ETM 305
OAc
CHO
CH3
H3C
200
250
300
350
400
0
.5
1
1.5
2

FIG. 9

UV SPECTRUM OF ETM (PHARMAMAR).

INT OF WINDOW 39: UV APEX SPECTRUM OF PEAK 7.82 OF PICO-M2.D

UV. APEX SPECTRUM OF PEAK 7.82 OF PICO-M2.D

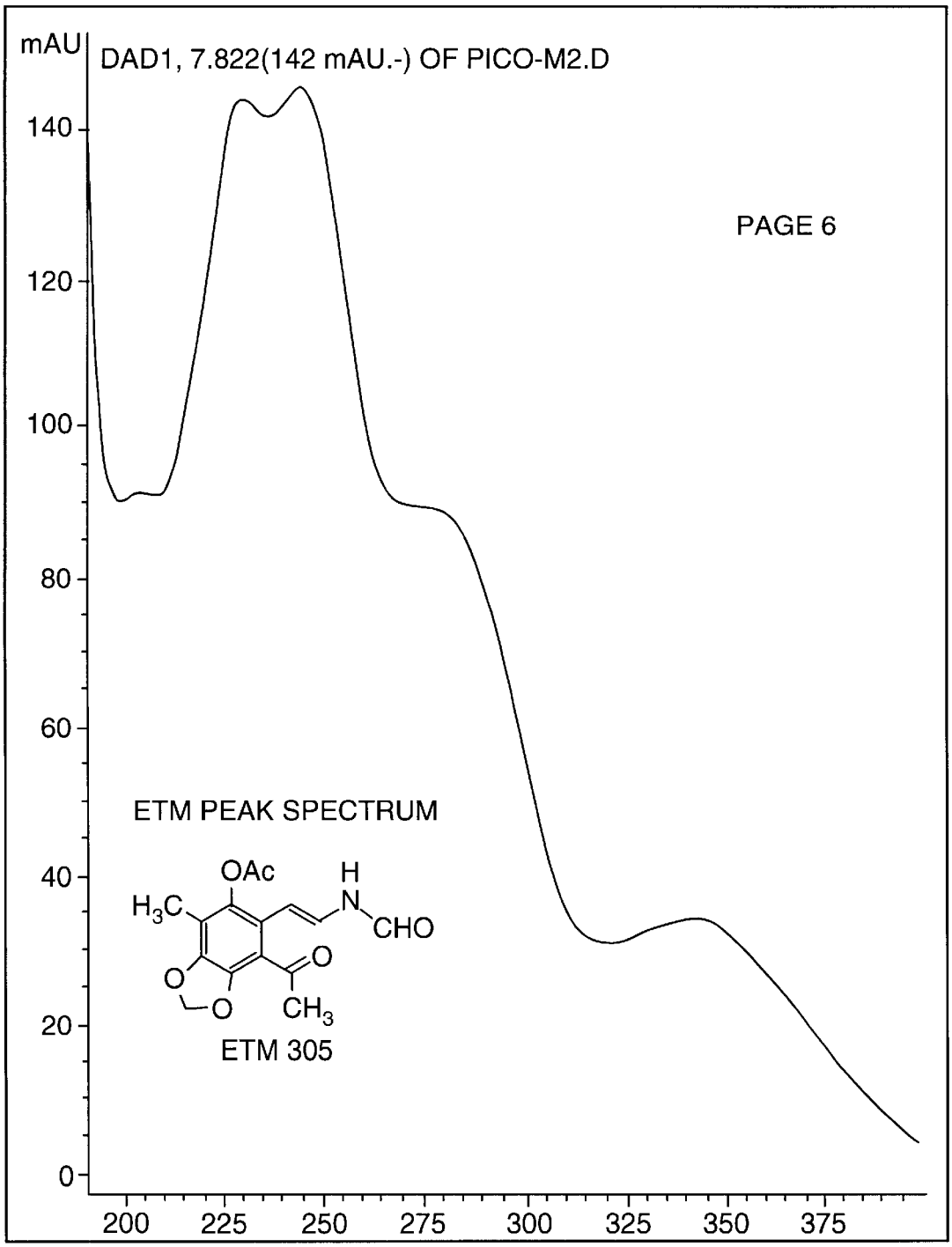


FIG. 10

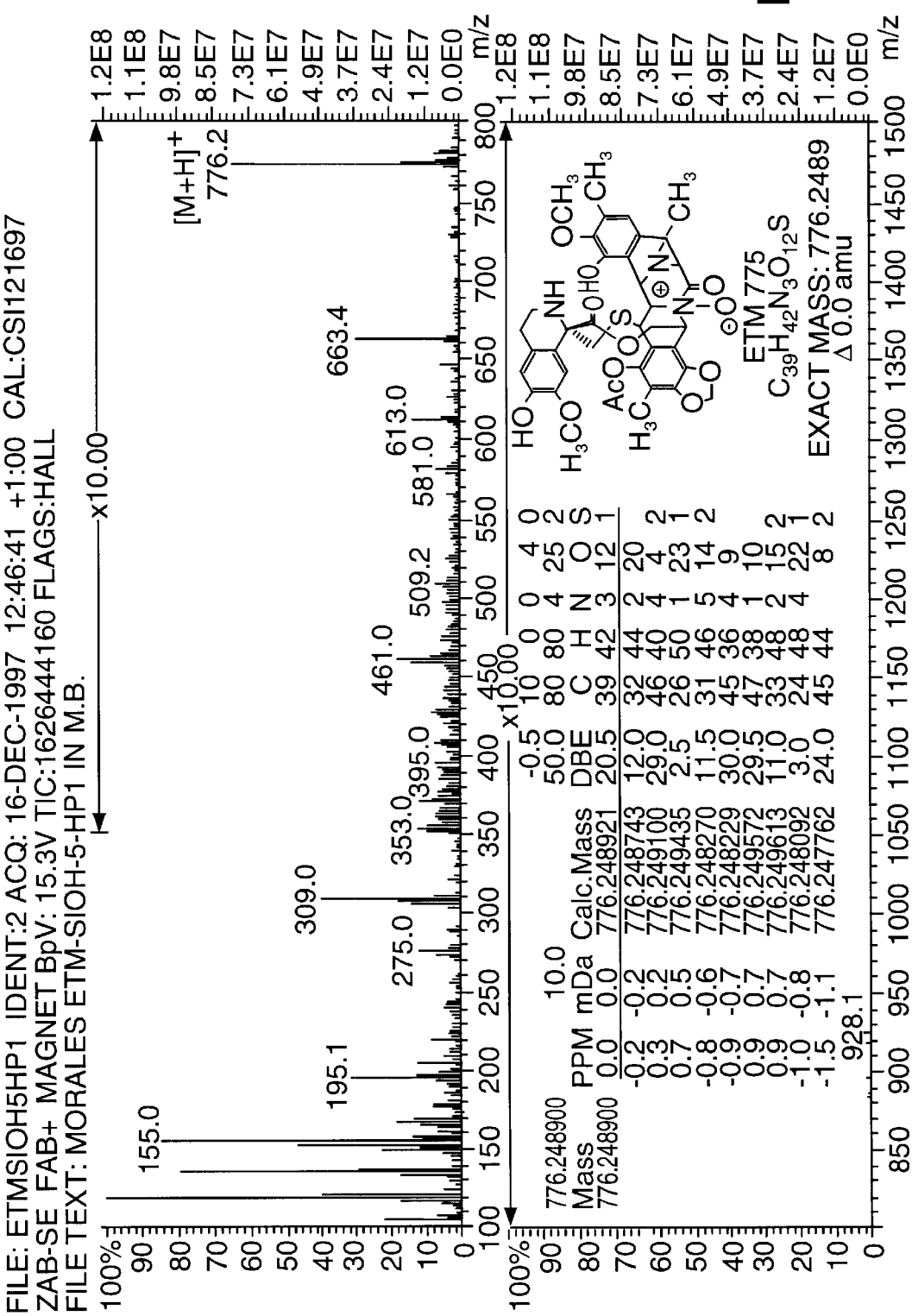

LRFAB MASS SPECTRUM OF ETM 775 IN M.B.
FILE: ETMSIOH5HP1 IDENT:2 ACQ: 16-DEC-1997 12:46:41 +1:00 CAL:CSI121697
ZAB-SE FAB+ MAGNET BpV: 15.3V TIC:1626444160 FLAGS:HALL
FILE TEXT: MORALES ETM-SIOH-5-HP1 IN M.B.
x10.00
[M+H]+
776.2
663.4
613.0
581.0
509.2
461.0
395.0
353.0
309.0
275.0
195.1
155.0
m/z
ETM 775
C39H42N3O12S
EXACT MASS: 776.2489
Δ 0.0 amu
776.248900
Mass
776.248900
PPM mDa Calc.Mass DBE C H N O S
928.1

FIG. 11

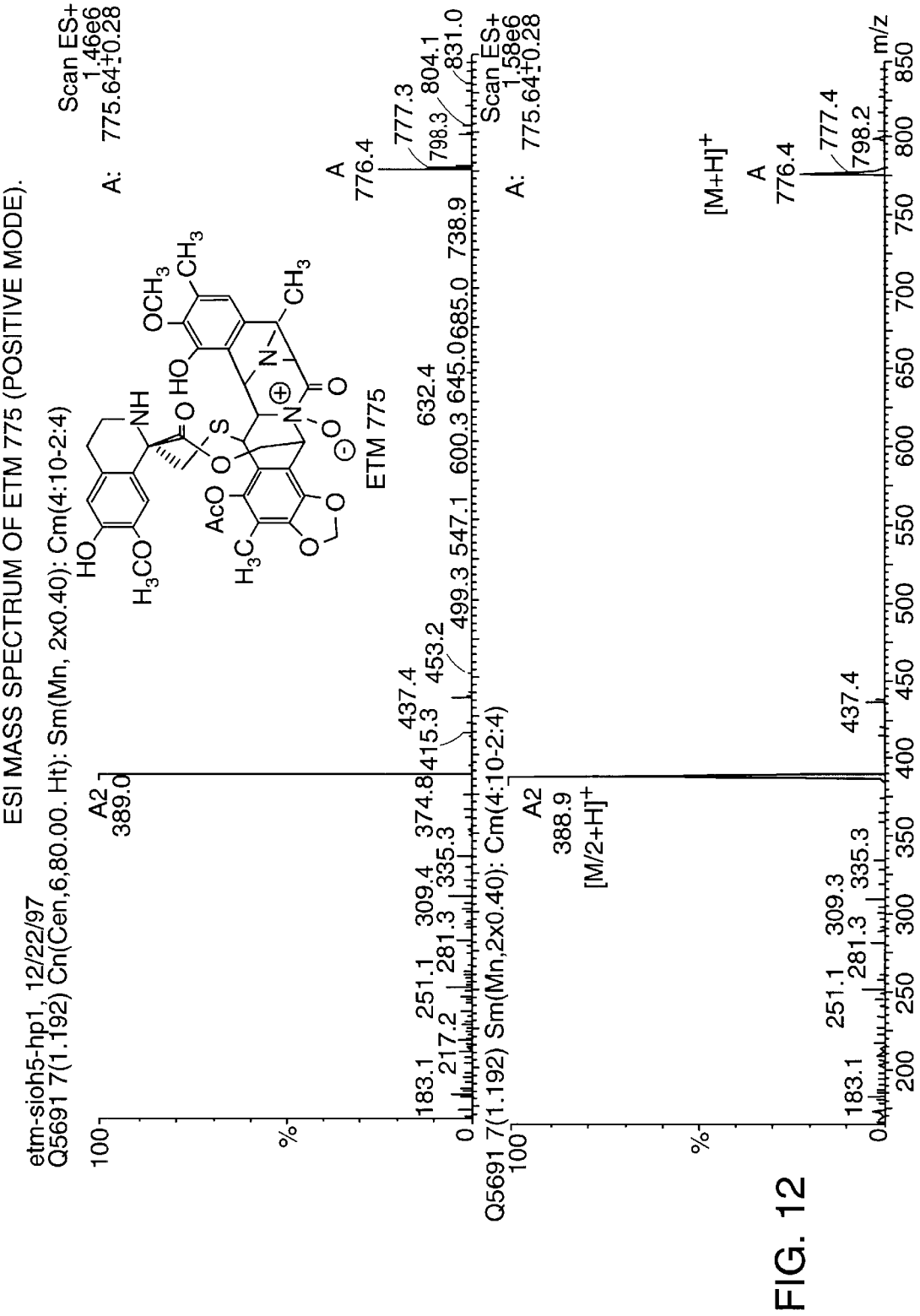

ESI MASS SPECTRUM OF ETM 775 (POSITIVE MODE).
etm-sioh5-hp1, 12/22/97
Q5691 7(1.192) Cn(Cen,6,80.00. Ht): Sm(Mn, 2x0.40): Cm(4:10-2:4)
Scan ES+
1.46e6
A: 775.64±0.28
ETM 775
Q5691 7(1.192) Sm(Mn,2x0.40): Cm(4:10-2:4)
Scan ES+
1.58e6
A: 775.64±0.28
[M+H]+
[M/2+H]+
m/z

FIG. 12

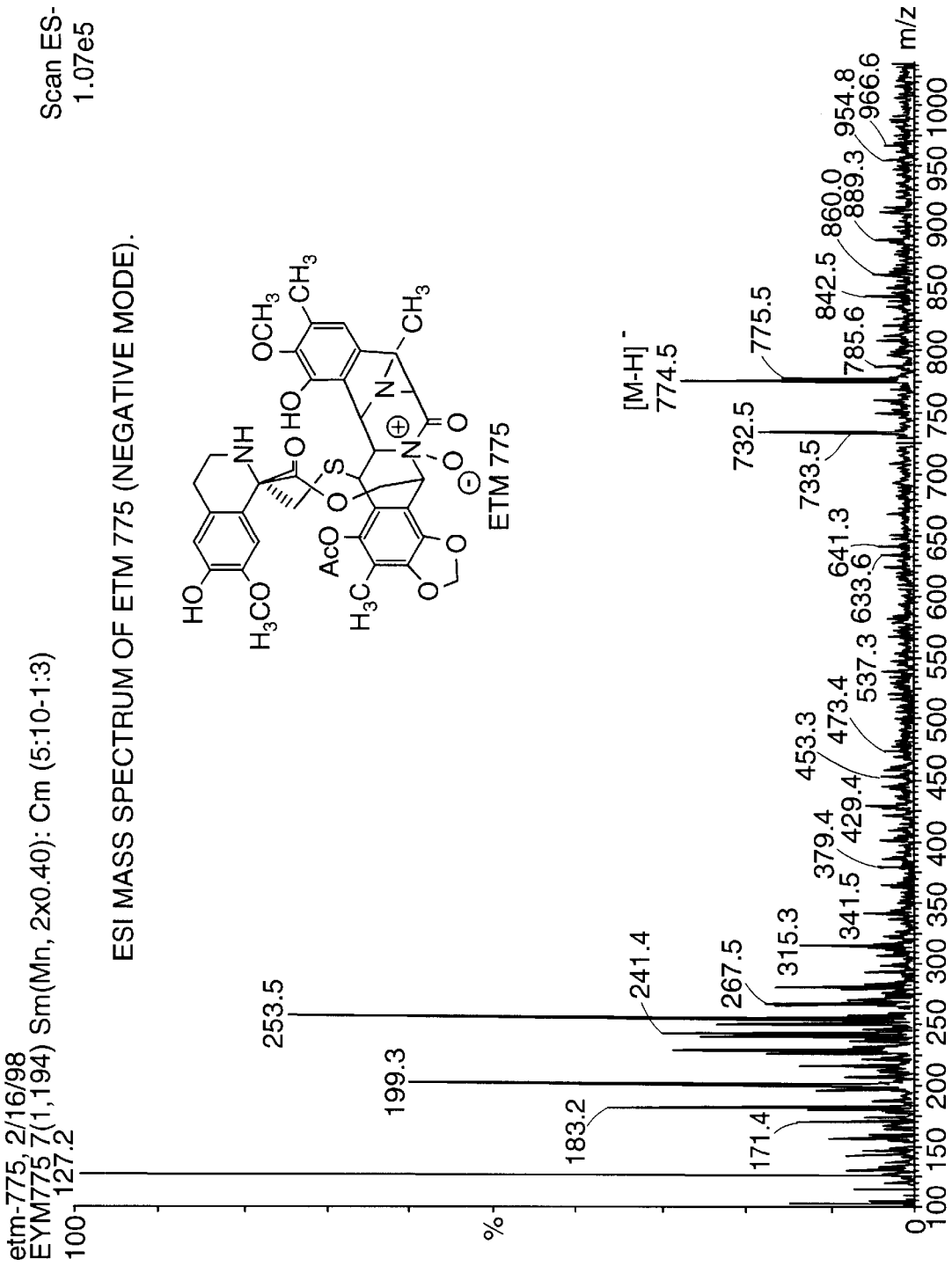

etm-775, 2/16/98
EYM775 7(1,194) Sm(Mn, 2x0.40): Cm (5:10-1:3)
Scan ES-
1.07e5
ESI MASS SPECTRUM OF ETM 775 (NEGATIVE MODE).
ETM 775
[M-H]⁻
774.5
775.5
732.5
733.5
842.5
860.0
889.3
954.8
966.6
785.6
641.3
633.6
537.3
473.4
453.3
429.4
379.4
341.5
315.3
267.5
241.4
253.5
199.3
183.2
171.4
127.2
100
%
0
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
m/z

FIG. 13

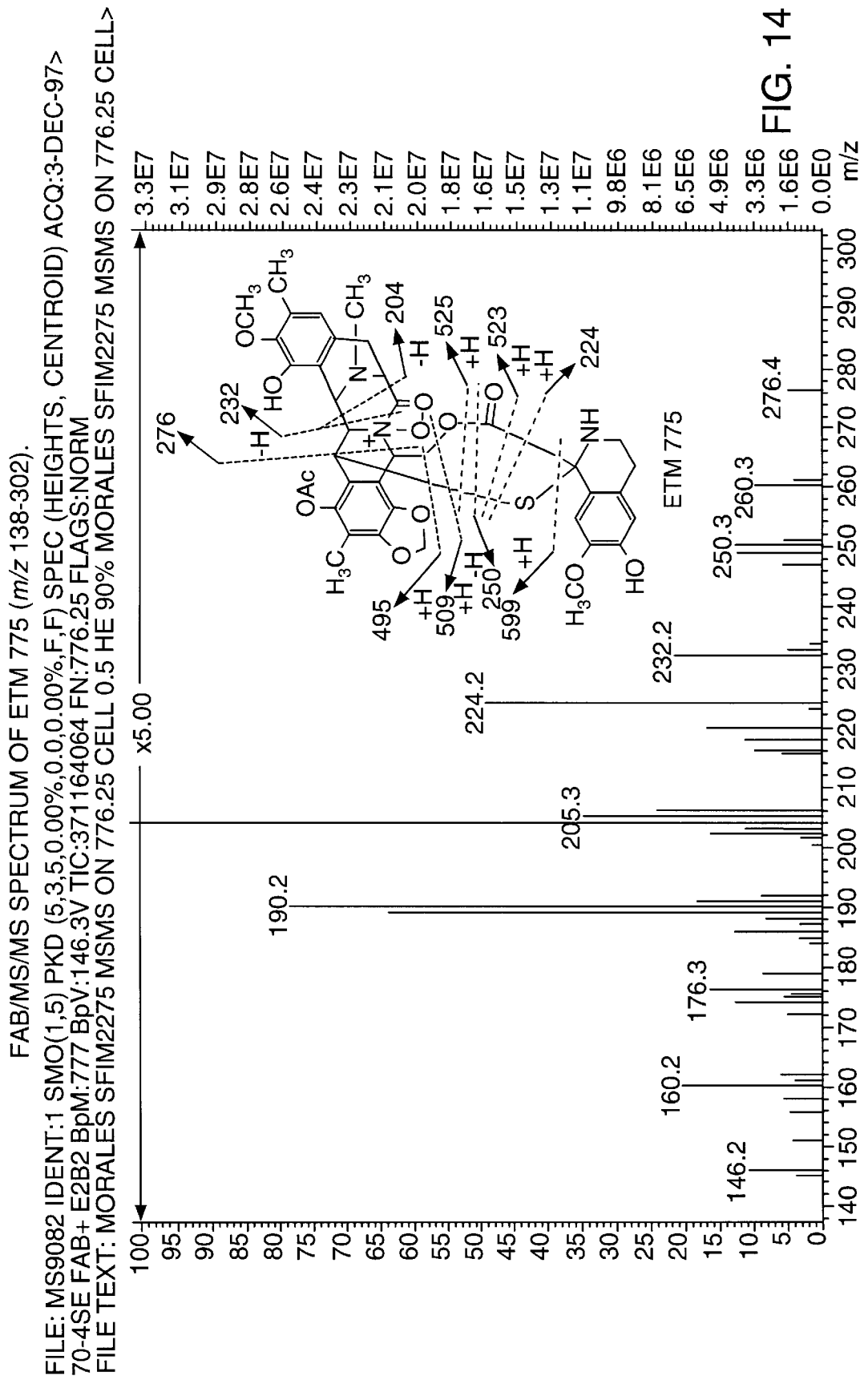

FAB/MS/MS SPECTRUM OF ETM 775 (m/z 138-302).
FILE: MS9082 IDENT:1 SMO(1,5) PKD (5,3,5,0.00%,0.0,0.00%,F,F) SPEC (HEIGHTS, CENTROID) ACQ:3-DEC-97>
70-4SE FAB+ E2B2 BpM:777 BpV:146.3V TIC:371164064 FN:776.25 FLAGS:NORM
FILE TEXT: MORALES SFIM2275 MSMS ON 776.25 CELL 0.5 HE 90% MORALES SFIM2275 MSMS ON 776.25 CELL>
x5.00
ETM 775
m/z

FIG. 14

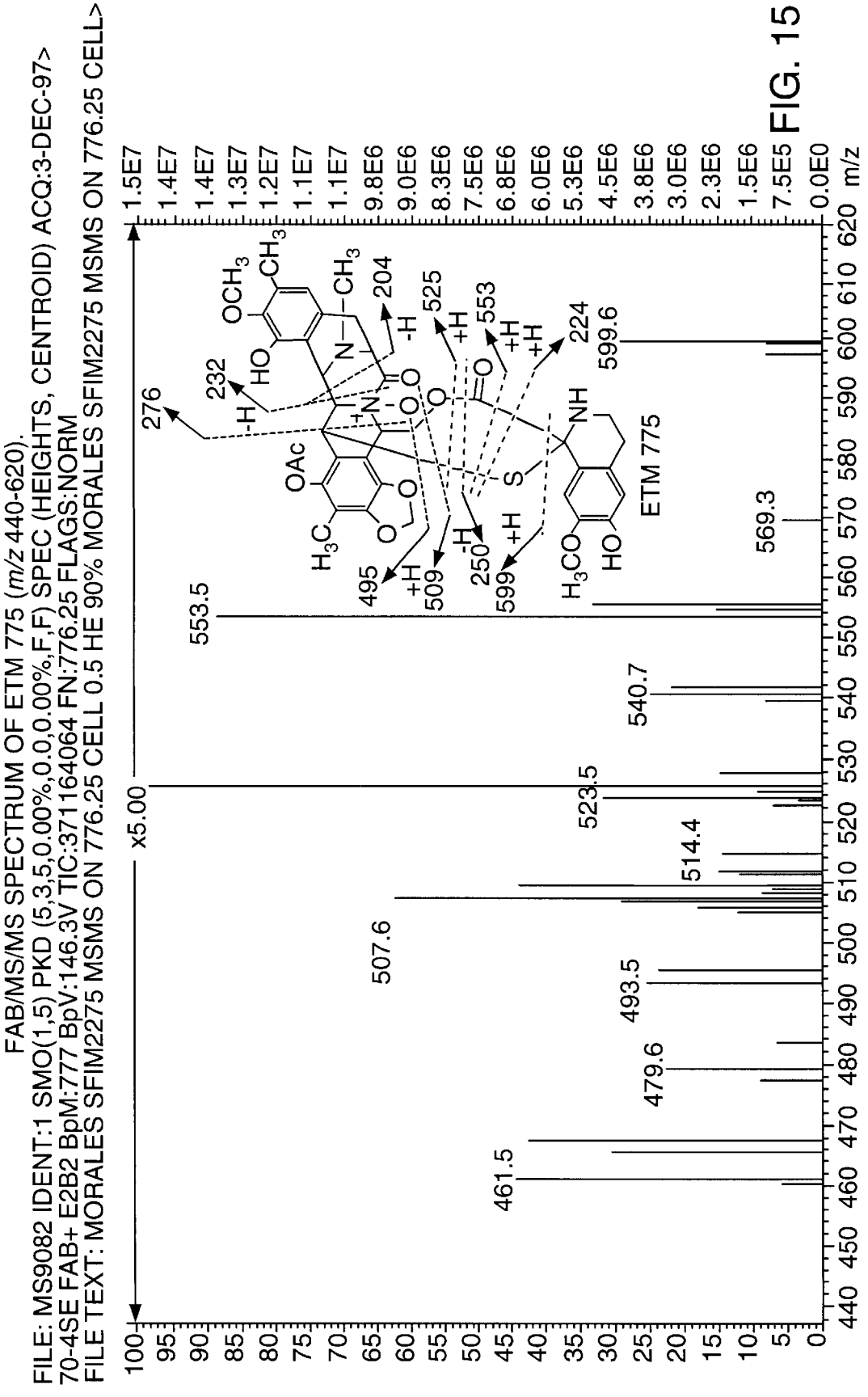

FAB/MS/MS SPECTRUM OF ETM 775 (m/z 440-620).
FILE: MS9082 IDENT:1 SMO(1,5) PKD (5,3,5,0.00%,0.0,0.00%,F,F) SPEC (HEIGHTS, CENTROID) ACQ:3-DEC-97>
70-4SE FAB+ E2B2 BpM:777 BpV:146.3V TIC:371164064 FN:776.25 FLAGS:NORM
FILE TEXT: MORALES SFIM2275 MSMS ON 776.25 CELL 0.5 HE 90% MORALES SFIM2275 MSMS ON 776.25 CELL>
x5.00
ETM 775
553.5
523.5
540.7
514.4
507.6
493.5
479.6
461.5
569.3
599.6
276
232
204
525
553
495
509
250
599
224
m/z

FIG. 15

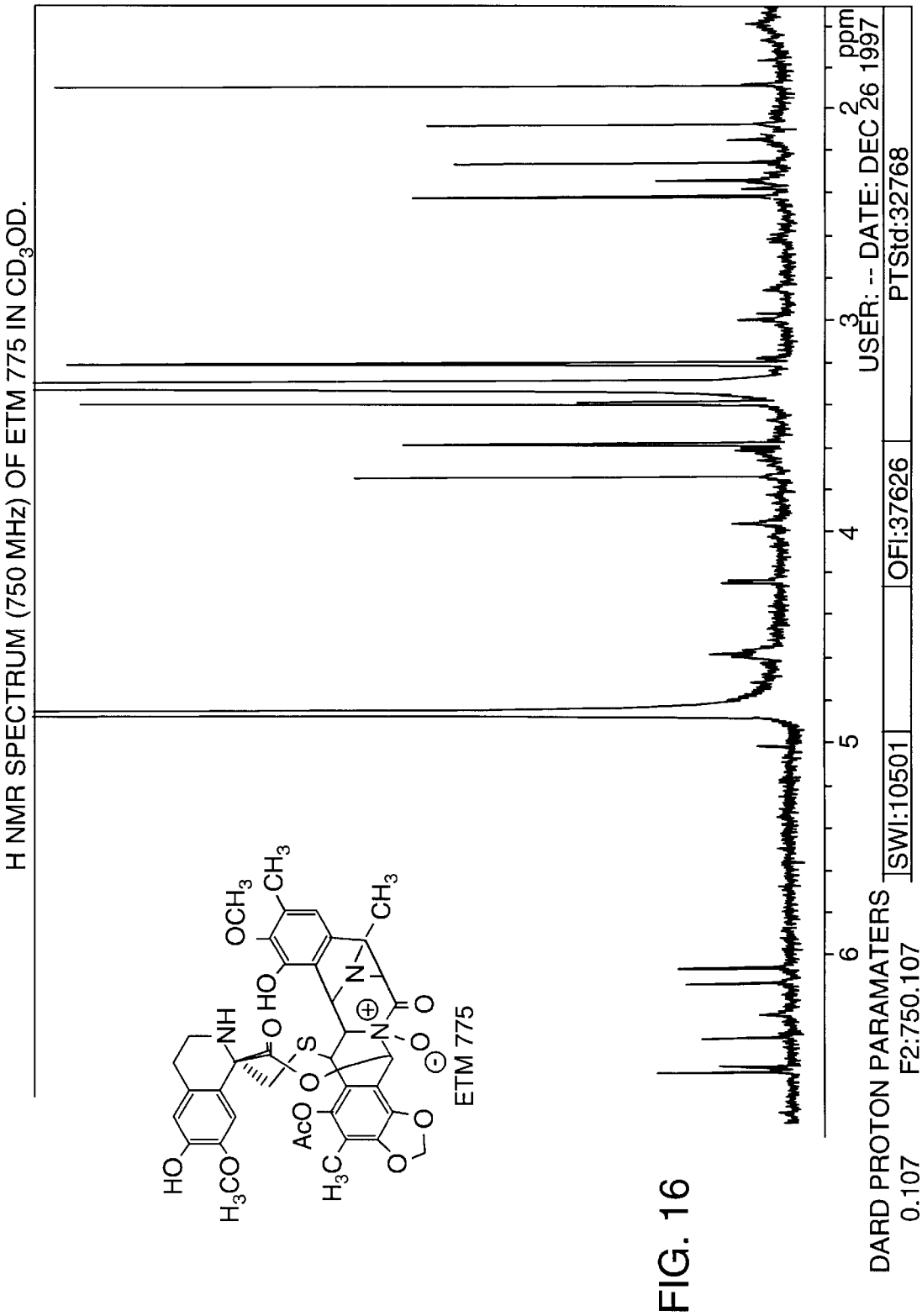
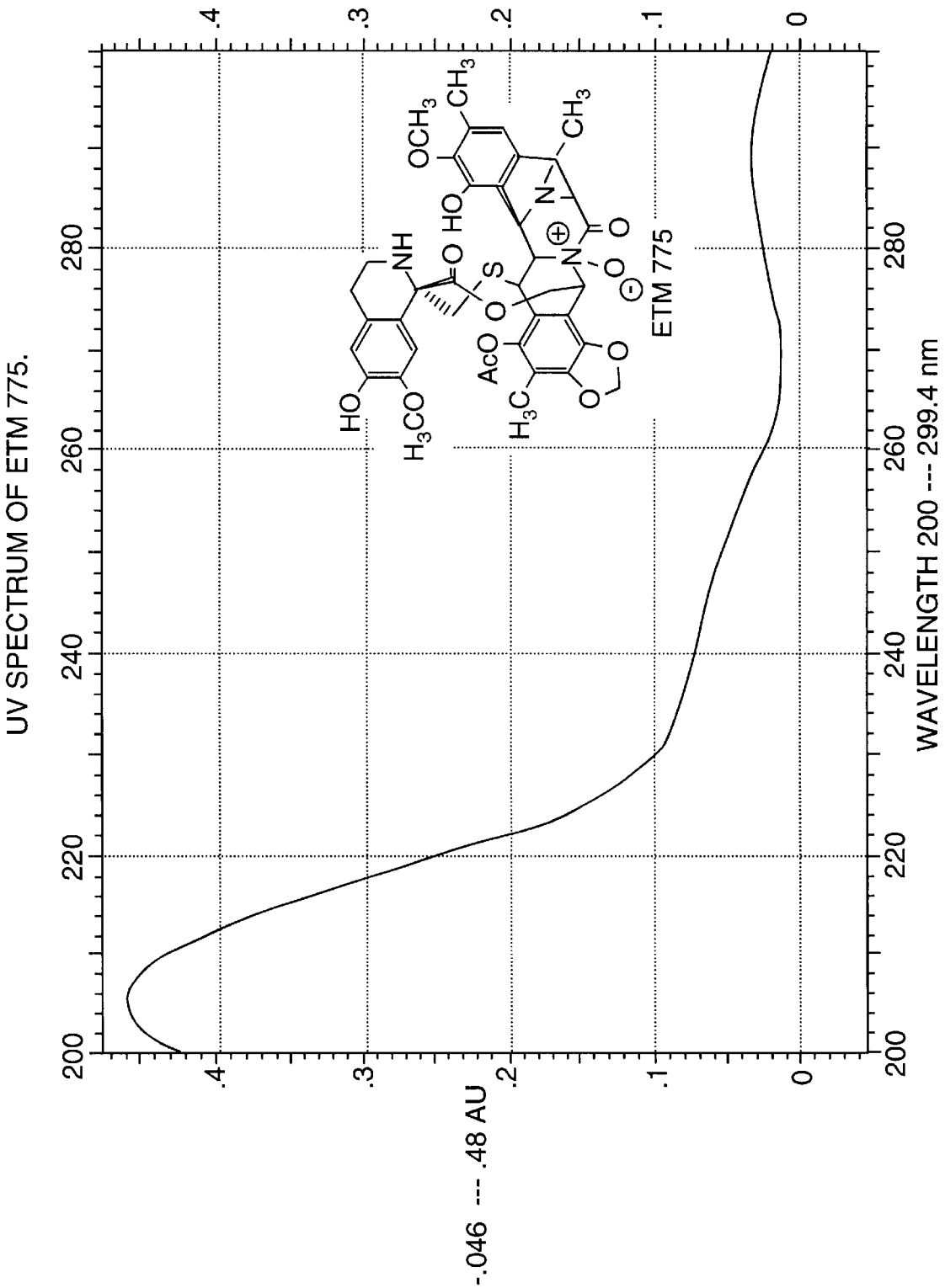

H NMR SPECTRUM (750 MHz) OF ETM 775 IN CD_3OD.
ETM 775
6
5
4
3
2
ppm
USER: -- DATE: DEC 26 1997
DARD PROTON PARAMATERS
SWI:10501
OFI:37626
PTStd:32768
0.107
F2:750.107

FIG. 16

FIG. 17


UV SPECTRUM OF ETM 775.
ETM 775
-.046 --- .48 AU
WAVELENGTH 200 --- 299.4 nm
200
220
240
260
280
0
.1
.2
.3
.4

HPLC CHROMATOGRAM OF M1 METABOLITE (ETM 305).

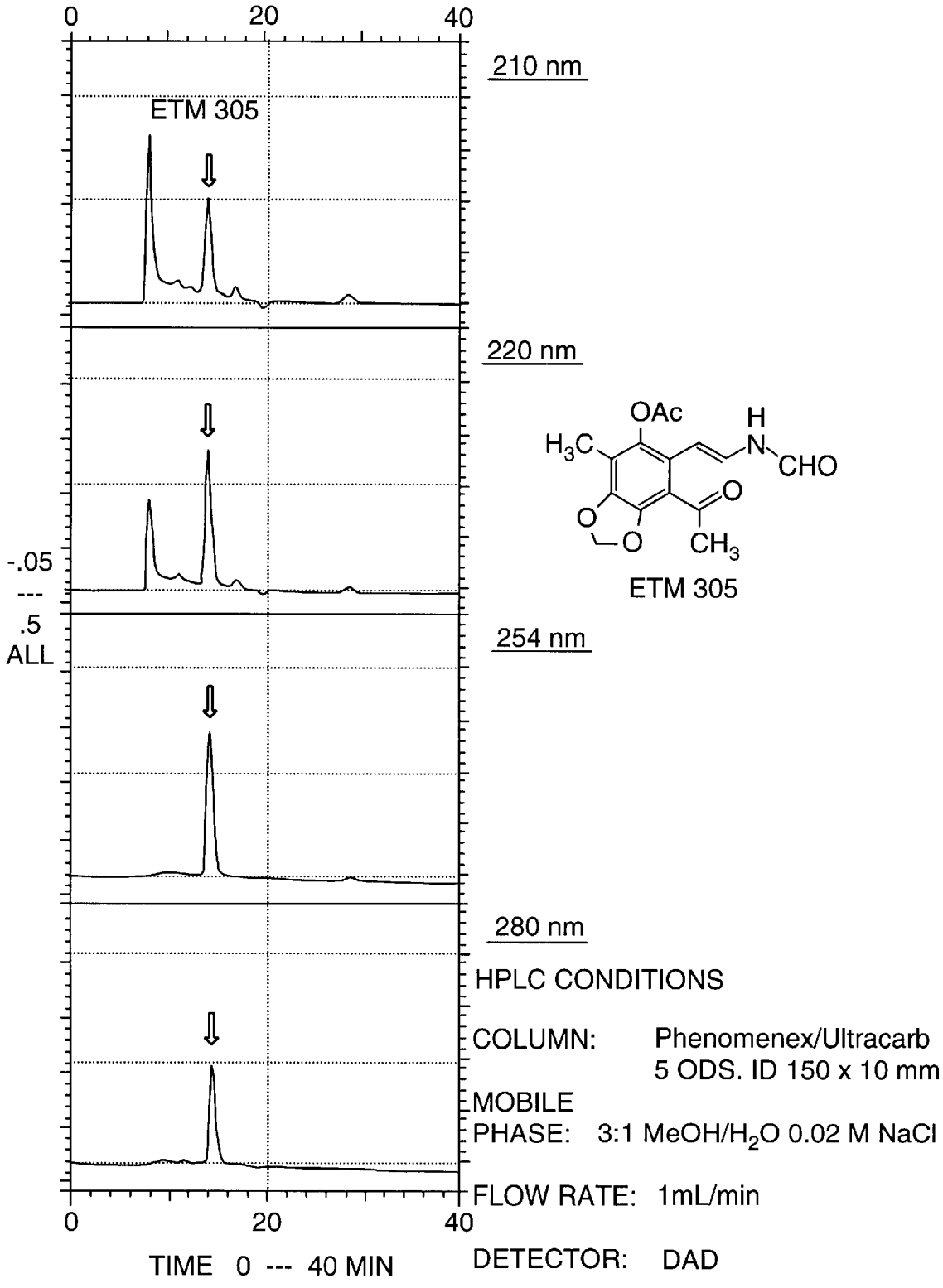


FIG. 18

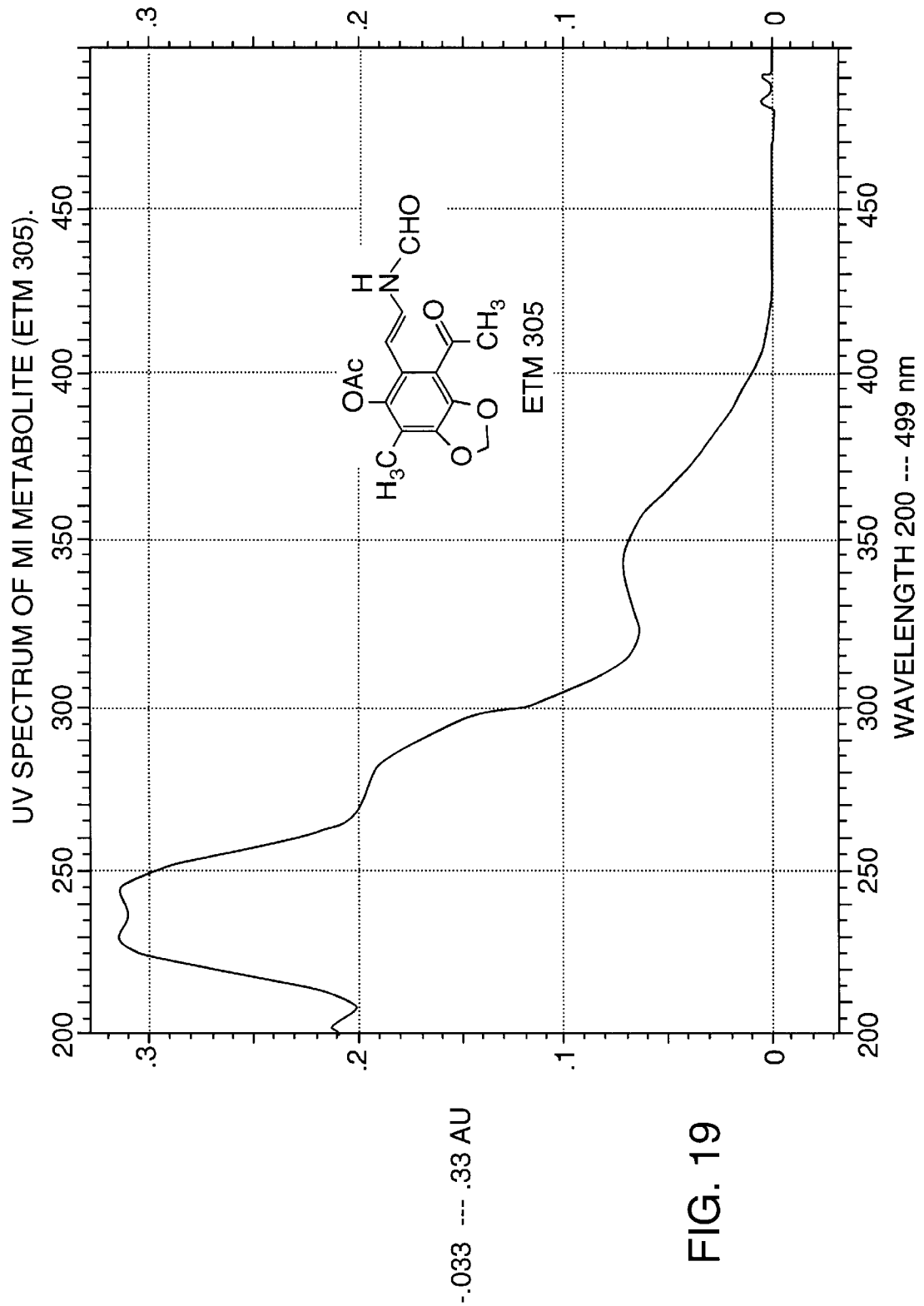

UV SPECTRUM OF MI METABOLITE (ETM 305).
-.033 --- .33 AU
WAVELENGTH 200 --- 499 nm
200
250
300
350
400
450
0
.1
.2
.3
ETM 305
OAc
H
N
CHO
O
CH3
H3C
O

FIG. 19

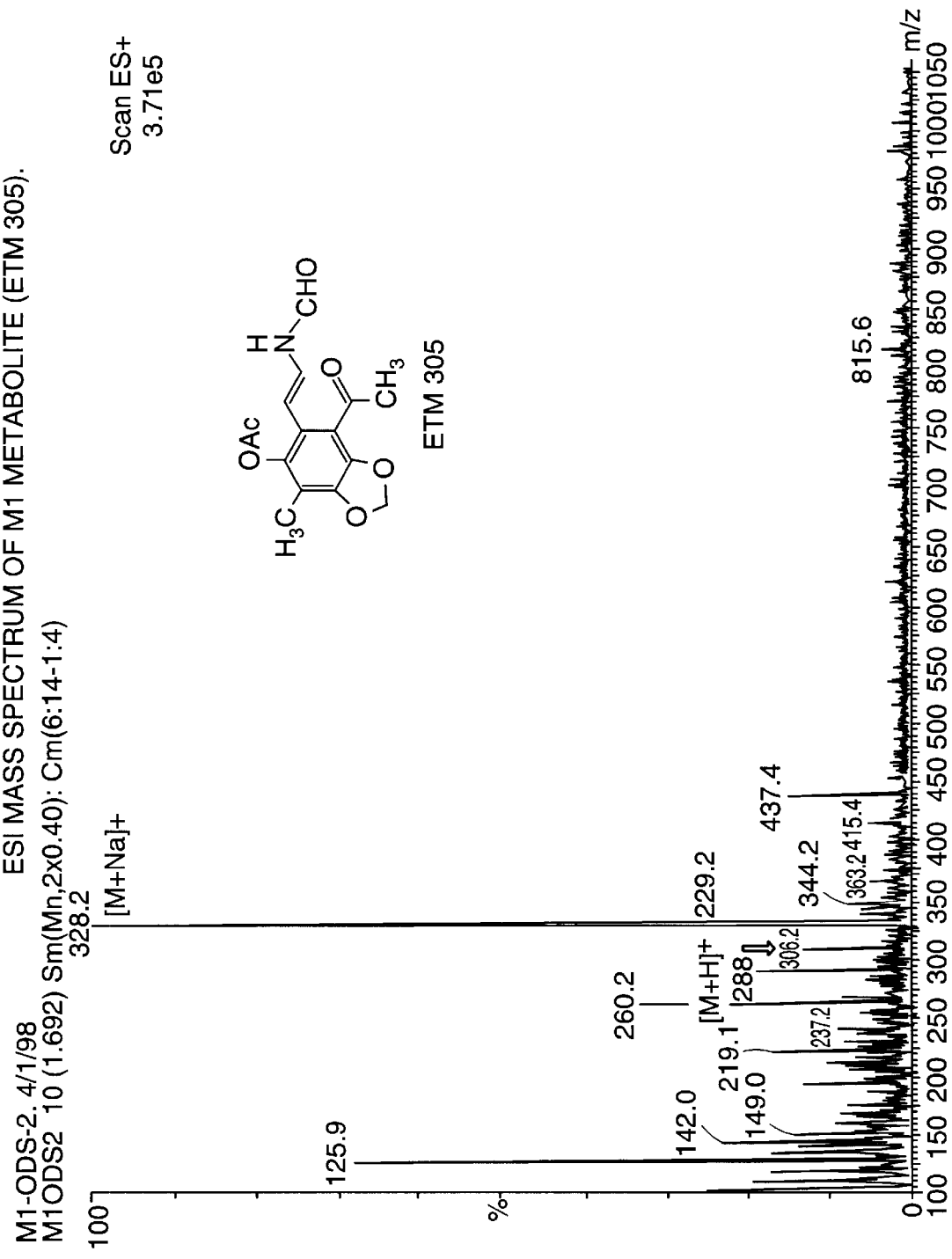

ESI MASS SPECTRUM OF M1 METABOLITE (ETM 305).
M1-ODS-2. 4/1/98
M1ODS2 10 (1.692) Sm(Mn,2x0.40): Cm(6:14-1:4)
Scan ES+
3.71e5
ETM 305
OAc
H
N
CHO
O
CH3
H3C
O
O
328.2
[M+Na]+
125.9
260.2
142.0
219.1
149.0
[M+H]+
288
306.2
237.2
229.2
437.4
344.2
363.2
415.4
815.6
%
100
0
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000 1050 m/z

FIG. 20

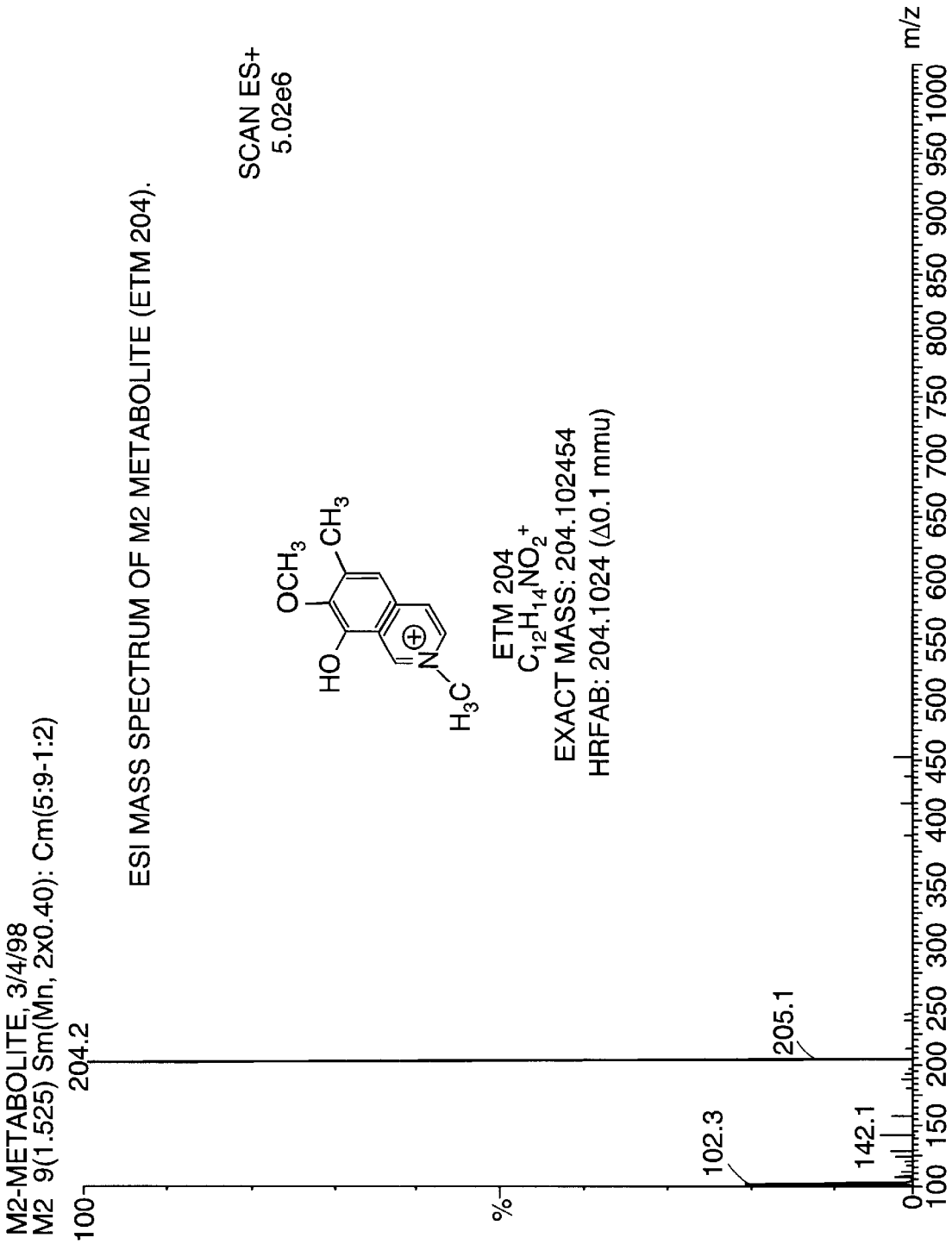

M2-METABOLITE, 3/4/98
M2 9(1.525) Sm(Mn, 2x0.40): Cm(5:9-1:2)
ESI MASS SPECTRUM OF M2 METABOLITE (ETM 204).
SCAN ES+
5.02e6
ETM 204
$C_{12}H_{14}NO_2^+$
EXACT MASS: 204.102454
HRFAB: 204.1024 (Δ0.1 mmu)
204.2
205.1
102.3
142.1
100
%
0
m/z
100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000

FIG. 21

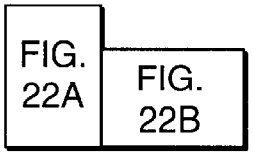


FIG. 22

MORALES, KLR, MZ IN CD3OD

EXPL S2PUL

SAMPLE	
DATE	MAR 17 98
SOLVENT	METHANOL
FILE	EXP
ACQUISITION	
STFRQ	499.701
TN	111
AT	4.003
NP	48000
SW	5996.1
FB	3400
BS	16
TPWR	63
PW	4.5
DL	0
TOF	0
NT	3000
CT	1044
ALOCK	N
GAIN	NOT USED
FLAGS	
11	N
LN	N
DP	Y
HS	NN
DISPLAY	
SP	- 0.1
WP	4997.0
V$	31752
SC	0
WC	250
HZMM	19.99
LS	33.57
RFL	2154.5
RFP	1649.0
TH	7
INS	1.000
NM	PH

DEC. & VT	
DFRQ	499.701
DN	H1
DPWR	20
DOF	0
DM	NNN
DMM	C
DMF	200
DSEQ	
DRES	1.0
HOMO	N
DEC2	
DFRQ2	0
DN2	
DPWR2	1
DOF2	0
DM2	N
DMM2	C
DMF2	200
DSEQ2	
DRES2	1.0
HOMO2	N
PROCESSING	
LB	0.30
WTFILE	
PROC	FT
FN	NOT USED
MATH	F
WERR	
WEXP	
WBS	
WNT	

FIG. 22A

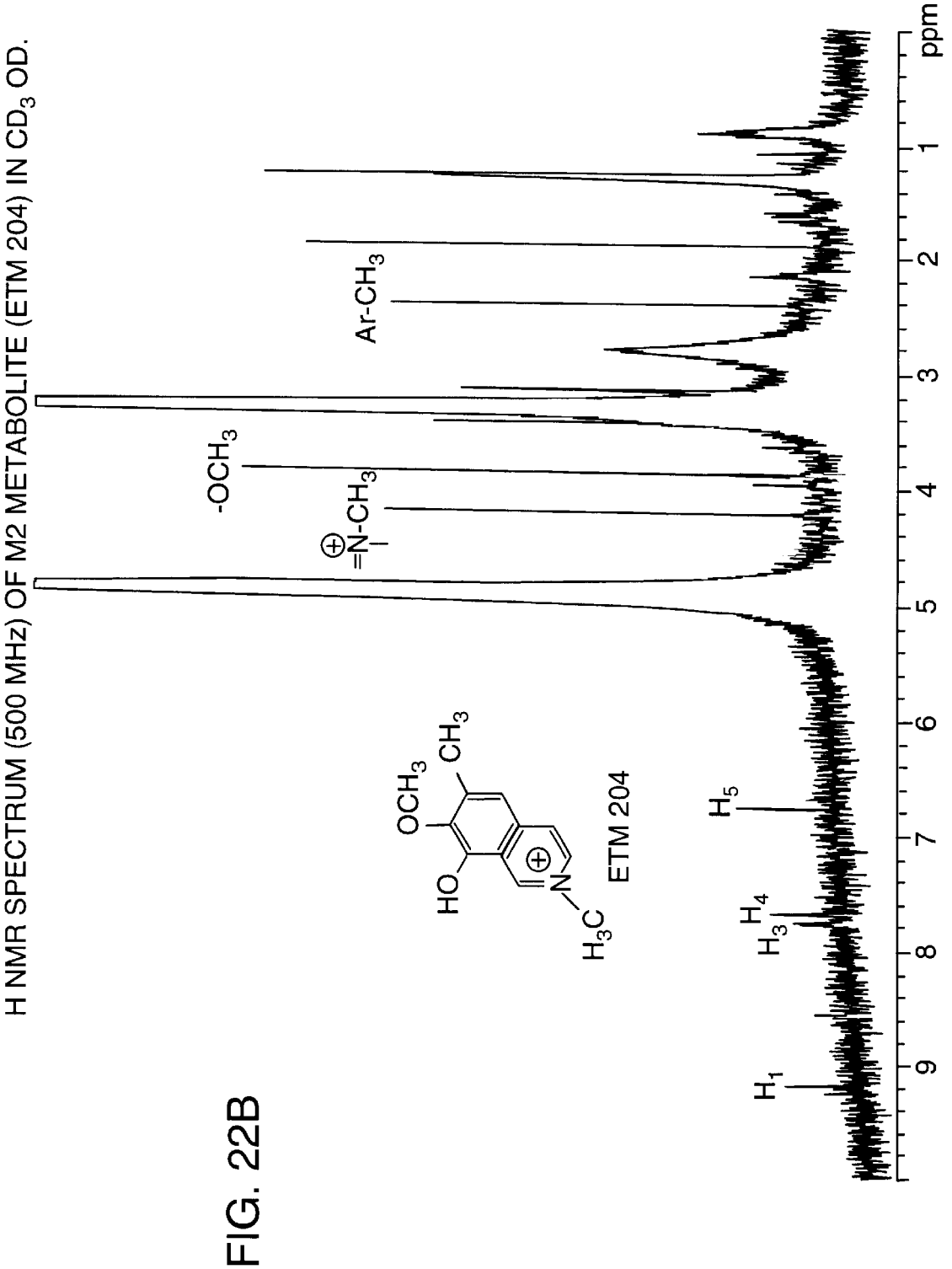

H NMR SPECTRUM (500 MHz) OF M2 METABOLITE (ETM 204) IN CD_3 OD.
Ar-CH_3
-OCH_3
$\oplus$ =N-CH_3
OCH_3
CH_3
HO
H_3C
ETM 204
H_5
H_4
H_3
H_1
ppm
1
2
3
4
5
6
7
8
9

FIG. 22B

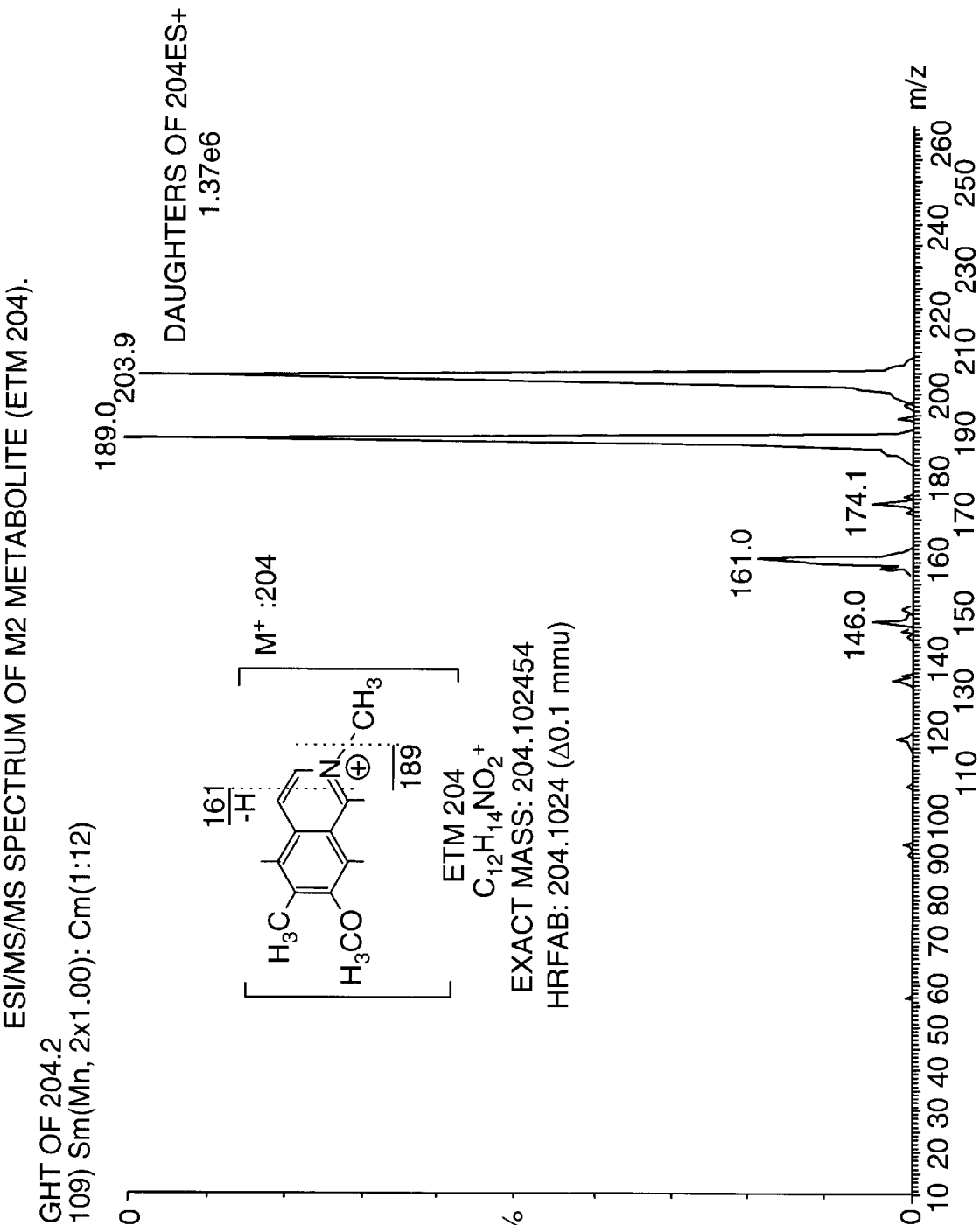

ESI/MS/MS SPECTRUM OF M2 METABOLITE (ETM 204).
M1, DAUGHT OF 204.2
M2D 1(0.109) Sm(Mn, 2x1.00): Cm(1:12)
DAUGHTERS OF 204ES+
1.37e6
189.0
203.9
161.0
174.1
146.0
M+ :204
161
-H
189
CH3
H3C
H3CO
ETM 204
C12H14NO2+
EXACT MASS: 204.102454
HRFAB: 204.1024 (Δ0.1 mmu)
100
%
0
10 20 30 40 50 60 70 80 90100 110 120 130 140 150 160 170 180 190 200 210 220 230 240 250 260
m/z

FIG. 23

ETM-775 METABOLITE OF ECTEINASCIDIN 743

CROSS-REFERENCE TO RELATED APPLICATION

This application claims priority benefit from copending U.S. Provisional Application Ser. No. 60/085,024, filed May 11, 1998, the disclosure of which is hereby incorporated herein by reference.

BACKGROUND OF THE INVENTION

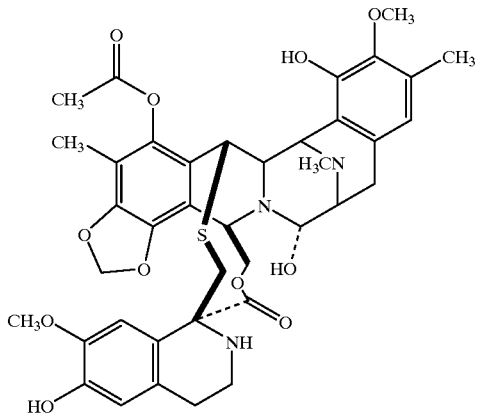
The ecteinascidins (herein abbreviated Et or Et's) are exceedingly potent antitumor agents isolated from the marine tunicate *Ecteinascidia turbinata.* In particular, Et's 729, 743 and 722 have demonstrated promising efficacy in vivo, including activity against P388 murine leukemia, B16 melanoma, Lewis lung carcinoma, and several human tumor xenograft models in mice.

The isolation and characterization of natural Et 743 is taught in U.S. Pat. No. 5,089,273 which is hereby incorporated herein by reference. The preparation of synthetic Et 743 is taught in U.S. Pat. No. 5,721,362, which is hereby incorporated herein by reference.

The antitumor activities of ecteinascidin compounds, particularly Et 729 and Et 743 are well documented in the scientific literature. See for example, Goldwasser et al., *Proceedings of the American Association for Cancer Research,* 39: 598 (1998); Kuffel et al., *Proceedings of the American Association for Cancer Research,* 38: 596 (1997); Moore et al., *Proceedings of the American Association for Cancer Research,* 38: 314 (1997); Mirsalis et al., *Proceedings of the American Association for Cancer Research,* 38: 309 (1997); Reid et al., *Cancer Chemotherapy and Pharmacology,* 38: 329–334 (1996); Faircloth et al., *European Journal of Cancer,* 32A, Supp. 1, pp. S5 (1996); Garcia-Rocha et al., *British Journal of Cancer,* 73: 875–883 (1996); Eckhardt et al., *Proceedings of the American Association for Cancer Research,* 37: 409 (1996); Hendriks et al., *Proceedings of the American Association for Cancer Research,* 37: 389 (1996); the disclosures of which are hereby incorporated herein by reference.

Ecteinascidin 743 (Et 743) has the following structure:

Et 743



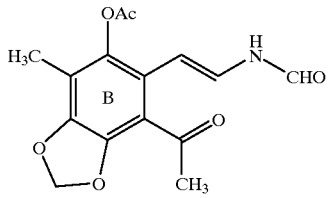
In view of the impressive antitumor activities of this class of compounds, the search continues for related structures that may possess equal or higher levels of antitumor activity. The present invention, which is directed to the isolation and characterization of natural metabolites of Et 743, is a result of these continued studies.

SUMMARY OF THE INVENTION

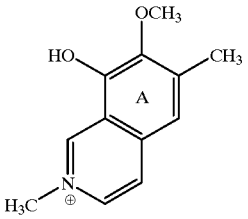
The purification and structure elucidation of several products of the metabolism of Et 743 by human cytochrome CYP3A4 have been accomplished. These compounds are abbreviated herein as "ETM" followed by a numeric value which represents the approximate molecular weight.

For example, ETM 305 and ETM 775 were isolated from a metabolic mixture obtained from a biochemical study performed by the Analytical Chemistry Department at PharmaMar, Spain. A similar metabolic study carried out by the Mayo Clinic led to the identification of ETM 204. The structures of these ecteinascidin metabolites are as follows:

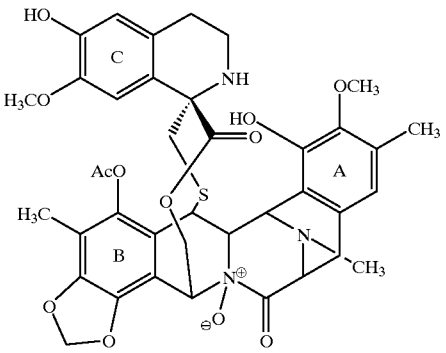
ETM 305



ETM 204



ETM 775



BRIEF DESCRIPTION OF THE DRAWINGS

The present invention may be better understood by reference to the drawings accompanying this specification, wherein:

FIG. **1** is the ^1H NMR spectrum (500 MHz) of ETM-SiOH-1 (non-polar impurity) in $CDCl_3$;

FIG. **2** is the HPLC chromatogram of ETM-SiOH-4 (ETM 775);

FIG. **3** is the HPLC chromatogram of ETM-SiOH-3 (ETM 305);

FIG. **4** is the HPLC chromatogram of ETM-SiOH-2 (trace metabolites);

FIG. **5** is the LRFAB mass spectrum of ETM 305 in M.B. (magic bullet[4]);

FIG. **6** is the ESI mass spectrum of ETM 305;

FIG. **7** is the ^{1}H NMR spectrum (750 MHz) of ETM 305 in CD_3OD;

FIG. **8** is the FAB/MS/MS spectrum of ETM 305;

FIG. **9** is the UV spectrum of ETM 305;

FIG. **10** is the UV spectrum of ETM;

FIG. **11** is the LRFAB mass spectrum of ETM 775 in M.B.;

FIG. **12** is the ESI mass spectrum of ETM 775 (positive mode);

FIG. **13** is the ESI mass spectrum of ETM 775 (negative mode);

FIG. **14** is the FAB/MS/MS spectrum of ETM 775 (m/z 138–302);

FIG. **15** is the FAB/MS/MS spectrum of ETM 775 (m/z 440–620);

FIG. **16** is the ^{1}H NMR spectrum (750 MHz) of ETM 775 in CD_3OD;

FIG. **17** is the UV spectrum of ETM 775;

FIG. **18** is the HPLC choromatogram of ETM 305;

FIG. **19** is the UV spectrum of ETM 305;

FIG. **20** is the ESI mass spectrum of ETM 305;

FIG. **21** is the ESI mass spectrum of ETM 204;

FIG. **22** is the ^{1}H NMR spectrum (500 MHz) of ETM 204 in CD_3OD; and

FIG. **23** is the ESI/MS/MS spectrum of ETM 204.

DETAILED DESCRIPTION OF THE INVENTION

I. Et 743 Metabolic Study

A. Preparation of Metabolic Mixture—ETM

Et-743 (50 μM) was incubated with 0.4 mg/ml of human lymphoblast-expressed CYP3A4 isoform (Gentest Corporation, Woburn, Mass.) in 0.1 M Tris-HCl buffer (pH 7.4) containing an NADPH generating system (0.4 mM $NADP^+$, 25 mM glucose-6-phosphate, 0.5 U/ml glucose-6-phosphate dehydrogenase and 3.3 mM magnesium chloride). After four (4) hours at 37° C., the reaction was stopped with ice cold acetonitrile and the solids removed by centrifugation (12,000 g, 4 min.). Supernatants were analyzed by HPLC.

B. Purification of ETM 305 and ETM 775

2.6 mg of ETM (generated as in A, above) was dissolved in a small amount of $CHCl_3$ and loaded into a silica gel column (8×100 mm glass column filled with a silica gel/$CHCl_3$ slurry). First, the column was eluted with $CHCl_3$ followed by $CHCl_3$/MeOH mixtures (98, 96, 94, 92 and 90%). A total of ten test tubes were collected (3 mL each) and combined on the basis of TLC to yield four fractions (Table 1). The less polar and non-cytotoxic fraction (ETM-SiOH-1, 2 mg) consisted of a lipid not structurally related to Et 743 as revealed by the ^{1}H NMR spectrum (FIG. **1**).

The remaining cytotoxic fractions were further purified by HPLC (Phenomenex-Ultracarb ODS, 10 μm, 10×150 mm, 3:1 MeOH/H_2O 0.02 M NaCl, 1 mL/min., Da Detection: 210, 220, 254 and 280 nm). The most polar fraction (ETM-SiOH-4, 0.2 mg) yield 0. 1 mg of ETM 775 (FIG. **2**). ETM-SiOH-3 yield 0.3 mg of ETM 305 (FIG. **3**), and ETM-SiOH-2 consisted of a complex mixture of trace metabolites (FIG. **4**).

TABLE 1

ETM-SiOH fractions: R_f, weight and cytoxic activity.

ID #	Test tube #	R_f[a]	Weight	L1210 growth inhibition (%) at 500 ng/mL
ETM-SiOH 1	1	0.9	2.0 mg	0
ETM-SiOH 2	2	0.5, 0.7	0.3 mg	80[b]
ETM-SiOH 3	4–5	0.5	0.4 mg	30
ETM-SiOH 4	6	0.3	0.2 mg	3

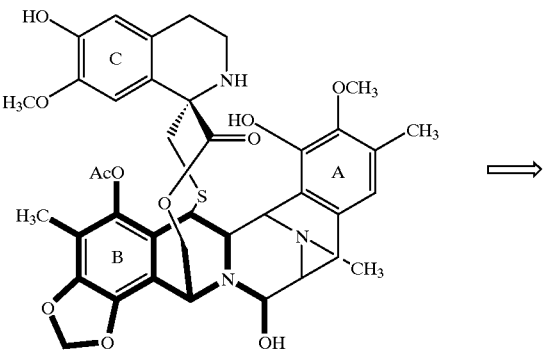
[a]Silica gel TLC using 9:1 $CHCl_3$/MeOH as mobile phase.
[b]30% inhibition at 250 ng.

C. The Structure of ETM 305

ETM 305 (IC_{50} 0.2 μm/mL vs L1210 cells) showed a molecular ion at 306.0977 by HRFAB/MS (FIG. **5**). This data is in agreement with the molecular formula $C_{15}H_{16}NO_6$ (Δ0.1 mmu). ESI/MS analysis confirmed the molecular weight of ETM 305 (FIG. **6**); a molecular ion at m/z 306 was observed together with its sodium adduct (m/z 328). The ^{1}H NMR spectrum of ETM 305 (FIG. **7**) was very important for the structural assignment. Resonances at δ2.04, 2.28 and 6.09 were almost identical to those of Me-6 (δ2.03), —$OCOCH_3$ (δ2.29) and the dioxy-methylene protons (δ6.11 and 6.01) in Et 743,[1] respectively.

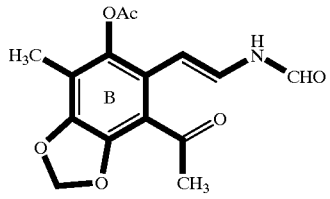
In addition, it was observed resonances corresponding to a —CH═CH—NHCHO unit (δ7.09, d, 1H, J=15 Hz; δ6.19, d, 1H, J=15 Hz; δ8.04, s, 1H),[2] and an additional methyl group (δ2.52, s, 3H). The chemical shift of this methyl group match pretty well wit that of the methyl group on acetophenone[3] (δ2.55). It is interesting to note that the ^{1}H NMR spectrum of ETM 305 consisted of two sets of resonances (4:1 ratio) due to rotational conformers around the —NH—CHO bond The ^{1}H NMR data together with the MS data suggested that ETM 305 had the B-unit aromatic ring system of Et 743 attached to a vinyl-formamide unit and to a methyl ketone as shown in Scheme 1. FAB/MS/MS on m/z 306 supported the proposed structure (FIG. **8**).

Scheme 1



Et 743

-continued

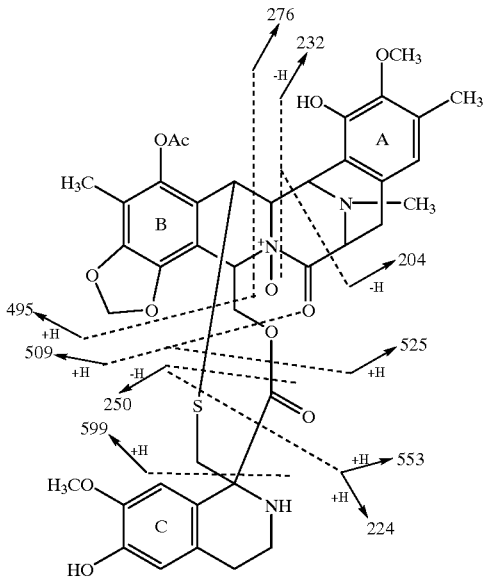


ETM 305
$C_{15}H_{15}NO_6$
Mol. Wt.: 305.28
HRFAB: 306.0977 (Δ 0.1 mmu)

D. The Structure of ETM 775

ETM 775 (IC_{50} 0.2 μg/mL vs L1210 cells) showed a molecular ion at 776.2489 by HRPAB/MS FIG. **11**). This data is in agreement with the molecular formula $C_{39}H_{42}N_3O_{12}S$ (Δ0.0 mmu) which indicated that ETM 775 is an oxidation product of Et 743. Both, positive and negative mode ESI/MS spectra confirmed the molecular weight of ETM 775 (FIGS. **12** and **13**). Because of the limited amount of ETM 775, the structural assignment was carried out mainly by interpretation of its mass spectral data. FABMS/MS on M+H of ETM 775 (m/z 776) was critical in assigning the location of the extra oxygen was located on N-2 in the form of an N-oxide as revealed by peaks at m/z 276 and 260 (276-oxygen). A fragment ion at m/z 232, not observed in Et 743, suggested that the carbinol amine oxygen was oxidized to the amide (Scheme 3). The structures of the A- and C-units in ETM 775 remained intact as revealed by the presence of the characteristic mass spectral peaks at m/z 204 (A-unit), and m/z 224 and 250 (C-unit).[1] Both, the 750 750 Mhz ^{1}H NMR (FIG. **16**) and the UV (FIG. **17**) spectra resembled those of Et 743.[1]

Scheme 2



ETM 775
$C_{39}H_{42}N_3O_{12}S$
HRFAB: 776.2489 (Δ 0.0 amu)

II. Et 743—Mayo Metabolic Study

A. M1 metabolite (ETM 305)

The ETM sample was filtered through a C18 sep-pack and the eluant (3:1 MeOH/H_2O) concentrated under a nitrogen stream. Purification of the resulting residue by HPLC (same conditions as described above) revealed the presence of a compound with a retention time identical to that of ETM 305 (FIG. **18**). Both, the UV (FIG. **19**) and ESI/MS (FIG. **20**) spectra of M1 were identical to that of ETM 305. Thus, it was concluded that M1 metabolite had the same chemical structure as ETM 305.

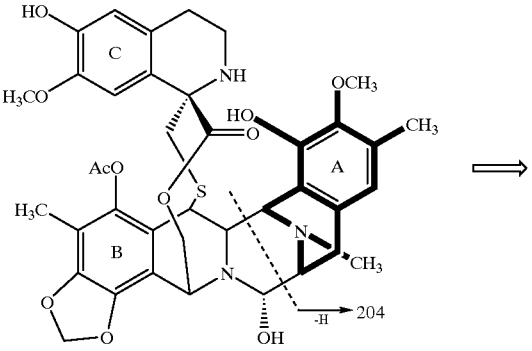
B. M2 metabolite (ETM 204)

The provided sample was filtered through a C18 sep-pack and the eluant (3:1 MeOH/H_2O) concentrated under a nitrogen stream and the resulting residue analyzed by FAB/MS, ESI/MS and ^{1}H NMR.

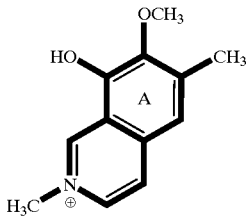
C. The Structure of ETM 204 (M2)

ETM 204 showed a molecular ion at 204.1024 by HRFAB/MS. This data is in agreement with the molecular formula $C_{12}H_{14}NO_2$ (Δ0.0 mmu). ESI/MS analysis confirmed the molecular weight as 204 (FIG. **21**). The molecular formula matched with the molecular formula of the a-unit in Et 743. Thus, the chemical structure of ETM 204 was proposed to be the aromatic ammonium salt derivative shown in Scheme 3. This simple compound (as well as the other metabolites) can easily be monitored to assay the breakdown of Et 743 in vivo.

Scheme 3



Et 743



ETM 204
$C_{12}H_{14}NO_2^+$
Mol. Wt.: 204.25
HRFAB: 204.1024 (Δ 0.0 mmu)

A ^{1}H NMR spectrum (FIG. **22**) of ETM 204 showed resonances that supported the proposed structure: four aromatics signals (δ9.2, s; δ7.8, d, J=5 Hz, and 6 6.8, s) and three methyl singlets (δ4.2, δ3.9 and δ2.4) The ESI/MS/MS of ETM 204 (FIG. **23**) showed a prominent peak ion at 189 corresponding to the apparent loss of the N-methyl group (204—CH_3).

Biological Studies of ETM-305 and ETM-775

Compounds ETM-305 and ETM-775 have been assayed employing standard protocols for the following tumor cell lines; P-388 (murine leukemia); A-549 (human lung carcinoma); HT-29 (human colon adenocarcinoma); and MEL-28 (human malignant melanoma). See, for example, Bergeron et al., *Biochem. Biophys. Res. Comm.,* 1984, 121 (3) 848–854 and Schroeder et al., *J. Med. Chem.,* 1981, 24 1078–1083. These results are shown below in Table 2:

TABLE 2

	Cell Line & Activity IC_{50} (μg/ml) Compound:			
	P-388	A-549	HT-29	MEL-28
ETM-305	0.5	0.5	0.5	0.25
ETM-775	0.01	0.01	0.01	0.01

Methods of Treatment

The present invention includes bioactive compounds, and accordingly, an embodiment of the present invention is directed to methods of treatment using such compounds. As described above, the compounds of the present invention have exhibited in vitro cytoxicity against tumor cell lines. It is anticipated that these in vitro activities will likewise extend to in vivo utility.

These compounds have been isolated in substantially pure form, i.e., at a purity level sufficient to allow physical and biological characterization thereof. These compounds have been found to possess specific antitumor activities and as such they will be useful as medicinal agents in mammals, particularly in humans. thus, another aspect of the present invention concerns pharmaceutical compositions containing the active compounds identified herein and methods of treatment employment such pharmaceutical compositions.

As described above, the active compounds of the present invention exhibit antitumor activity, thus, the present invention also provides a method of treating any mammal affected by a malignant tumor sensitive to these compounds, which comprises administering to the affected individual a therapeutically effective amount of an active compound or mixture of compounds, or pharmaceutical compositions thereof. The present invention also relates to pharmaceutical preparations, which contain as active ingredient one or more of the compounds of this invention, as well as the processes for its preparation.

Example of pharmaceutical compositions include any solid (tablets, pills, capsules, granules, etc.) or liquid (solutions, suspensions of emulsions) with suitable composition or oral, topical or parenteral administration, and they may contained the pure compound or in combination with any carrier of other pharmacologically active compounds. These compositions may need to be sterile when administered parenterally.

The terms "unit dose" as it pertains to the present invention refers to physically discrete units suitable as unitary dosages for animals, each unit containing a predetermined quantity of active material calculated to produce the desired antitumor effect in association with the required diluent; i.e., carrier, or vehicle. The specifications for the novel unit dose of this invention are dictated by and are directly dependent on (a) the unique characteristics of the active material and the particular antitumor effect to be achieved, and (b) the limitations inherent in the art of compounding such active material for antitumor use in animals.

Unit dosage forms are typically prepared from the frozen or dried active compound (or salts thereof) by dispersement in a physiologically tolerable (i.e., acceptable) diluent or vehicle such as water, saline or phosphate-buffered saline to form an aqueous composition. Such diluents are well known in the art and are discussed, for example, in Remington's Pharmaceutical Sciences, 16th Ed., Mack Publishing Company, Easton, Pa. (1980) at pages 1465–1467.

Dosage forms can also include an adjuvant as part of the diluent. Adjuvants such as complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA) and alum are materials well known in the art, and are available commercially from several sources.

The quantity of active compound to be administered depends, inter alia, on the animal species to be treated, the subject animal's size, the size of the tumor (if known), the type of tumor (e.g., solid) present, and the capacity of the subject to utilize the active compound. Precise amounts of active compound required to be administered depend on the judgment of the practitioner and are peculiar to each individual, particularly where humans are the treated animals. Dosage ranges, however, can be characterized by a therapeutically effective blood concentration and can range from a concentration of from about 0.01 μM to about 100 μM, preferably about 0.1 μM to 10 μM.

Suitable regimes for initial administration and booster injections are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain a therapeutically effective concentration in the blood are contemplated.

REFERENCES

The following background references are provided to assist the reader in understanding this invention. To the extent necessary, the contents are hereby incorporated herein by reference.

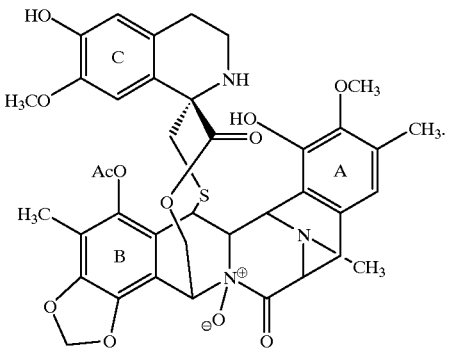
1. A) Rinehart et al., *J. Org. Chem.* 1990, 55, 4512. B) Rinehart et al., *J. Am. Chem. Soc.,* 1996, 118 9017.
2. Herbert et al., *J. Chem. Soc. Perkin Trans. I,* 1987, 1593.
3. Pretsch et al. *Tables of Spectral Data for Structure Determination of Organic Compounds;* Springer-Verla: Berlin, 1989; p. H125.
4. Rinehart et al., *Biochem. Res. Commun.,* 1984, 124, 350.

The present invention has been described in detail, including the preferred embodiments thereof However, it will be appreciated that those skilled in the art, upon consideration of the present disclosure, may make modifications and/or improvements on this invention and still be within the scope and spirit of this invention.

What is claimed is:

1. Substantially pure ETM-775 having the following structure:

ETM 775



2. A pharmaceutical composition comprising ETM-775 and a pharmaceutically acceptable carrier, diluent or excipient.

* * * * *