Electronic Supplementary Information (ESI)

Biosynthesis of violacein: a genuine intermediate, protoviolaceinic acid, produced by VioABDE, and insights into VioC function and the oxygenation mechanism of the central pyrrole ring.

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1. Construction of *vio*A, B, C, D, E

All the genes other than *vio*B were PCR-amplified by using a template of plasmid pVBG04, which was cloned by us (accession number AB032799). The following primers were used.

*vio*A:5'-GACATTCCATATGAAGCATTCTTCCGATATCTGC-3'
5'-TAAGGATCCTCACGCGGCGATGCGCTGCAGC-3'
*vio*C:5'-GAGGCCTCATATGAAAAGAGCAATAGTCGG-3'
5'-GAAGGATCCTCAGTTGACCCTCCCTATCTTG-3'
*vio*D:5'-GGGTCAACATATGAAGATTCTGGTCATCGGCG-3'
5'-GTAGGATCCTCAGCGTTGCAGCGCGTAGC-3'
*vio*E:5'-GAGGAGGCCATATGGAAAACCGGGAACCGC-3'
5'-GATCGGATCCCTAGCGCTTGGCGGCGAAG-3'

Nde Ⅰ and *Bam*H Ⅰ sites are underlined.

The gene *vio*B was amplified from plasmid pBaSa, which was prepared by digesting pVBG04 with *Bam*H1and *Sac*I, followed by the ligation into pUC119.

*vio*B:5'- GTTCGGGAAACATATGAGCATTCTGG -3'
5'- GAGCAATCATAGTCGGATCCGGGCTCGCCG -3'

All the PCR products of *vio*ABCDE were digested with *Nde* Ⅰ/*Bam*H Ⅰ and then ligated into pET3a (*vio*E) and pET12b (*vio*A,B,C, and D), which were transformed into *E. coli* BL21(DE3) for the overexpression of each protein. These plasmids were used in the cell-free experiments.

To purify the VioACDE with a Ni-NTA column, pET16b harboring *vio*A,C,D, and E and pET22b harboring *vio*B were constructed. These expressed proteins have His-tags.

The pET22b with *vio*B was constructed by PCR-amplification from plasmid pVBG04. The following primers were employed:

*vio*B:5'- CGGGAAACATATGAGCATTCTGGATTTTCCACGC-3'

5'- TCTTTTCAGAGCTCGCCTCTCTAGAAAGC-3'

The PCR products were digested with *Nde* Ⅰ/*Sac* Ⅰ and then ligated into pET22b. All the pET vectors thus constructed were transformed into *E. coli* BL21(DE3).

2. Culture and incubation conditions for the isolations of 8 and 9.

For the isolation of **8**, each of the cell-free extracts of Vio ABCDE was prepared as follows. *E. coli* BL 21 encoding each of the Vio ABCDE was grown at 37°C in 100 mL of Luria Broth medium with ampicillin (5 mg/ 100 ml). Expression of the recombinant protein was induced by adding 200 μl of 0.1M IPTG when the optical density at 600 nm reached about 0.5. The cultivation was continued for an additional 10 h at 25°C. To the pellets collected after the centrifugation, was added 20 ml of 0.2M NH_4Cl-buffer solution (pH 8.5), and then subjected to ultrasonication to disrupt cells. The supernatant was used as the enzyme source. The protein concentration of each enzyme solution were roughly estimated to be 0.6, 0.25, 0.58, 0.65 and 0.13 μM for VioA, B, C, D, E, respectively. These amounts were presumed by the calculation from the protein amounts that were recovered after the purification with Ni-NTA column, thus the values may not indicate the exact amounts of the expressed proteins. The combined cell-free solutions of VioABCD (83 ml each) were added to Trp (50 mg) and NADPH (150 mg) dissolved in 10 ml of NH_3-buffer solution (pH 8.5) and incubated for 1 h at 25°C on the rotary shaker at 120 rpm. The aerial condition (O_2) is necessary for the production of **1**. The biosynthetic intermediate **8** was extracted with EtOAc (250 ml), which quickly dried over Na_2SO_4 and evaporated into a small volume, but never dried (dryness causes the decomposition), followed by a column chromatography over SiO_2 with100% EtOAc. To examine which fraction contained **8**, a portion of each fraction, separated by the SiO_2 column, was incubated with the cell-free extract of VioC. The fractions giving **1** (bluish color) were combined, followed by HPLC (C_{18}) with MeOH:H_2O (45:55). The peaks were monitored at 254 nm. Next procedure for the NMR measurement was described in the main text. For the isolation of **9**, the mixed cell-free system of VioABE was used. Other procedures were the same as those of **8**, but no NADPH was added to the VioABE mixture, because NADPH is not required for the enzyme activities of VioA, VioB and VioE. HPLC (C_{18}) was carried out with MeOH:20mM NH_3-buffer (pH 8.5)=45:55. The retention times of **8** and **9** with this NH_3-buffer system was significantly shorter,

compared to those with MeOH:H_2O (45:55) (Fig. S1), further indicating that **8** and **9** involve a carboxyl group. Fig. S1 shows the HPLC (C_{18}) profiles of the EtOAc-extract from the incubation mixture by VioABDE. VioD is a monooxygenase that hydroxylate at 6-position of **9** to afford **8**. If a sufficient amount of VioD exists, no production of **9** would have been found, that is, **8** only would have been produced.

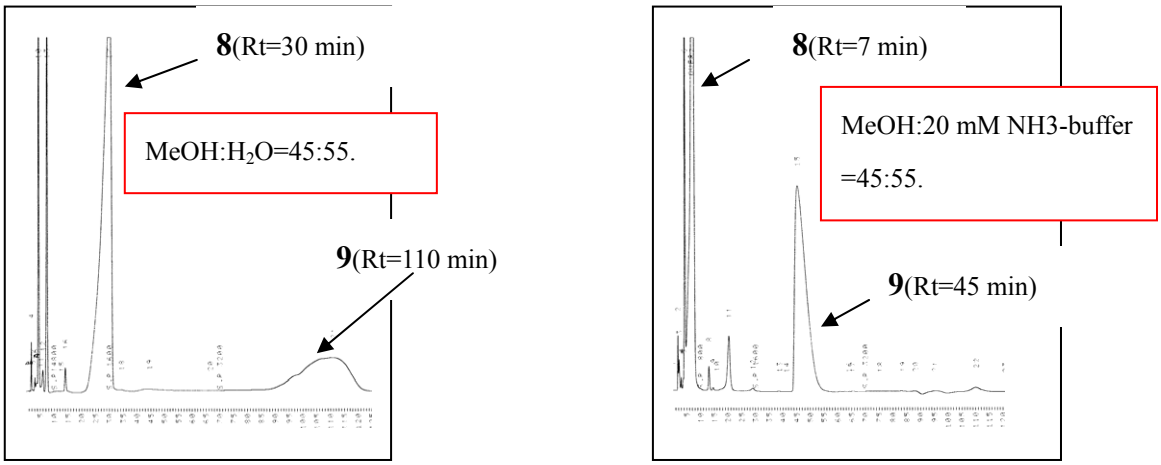
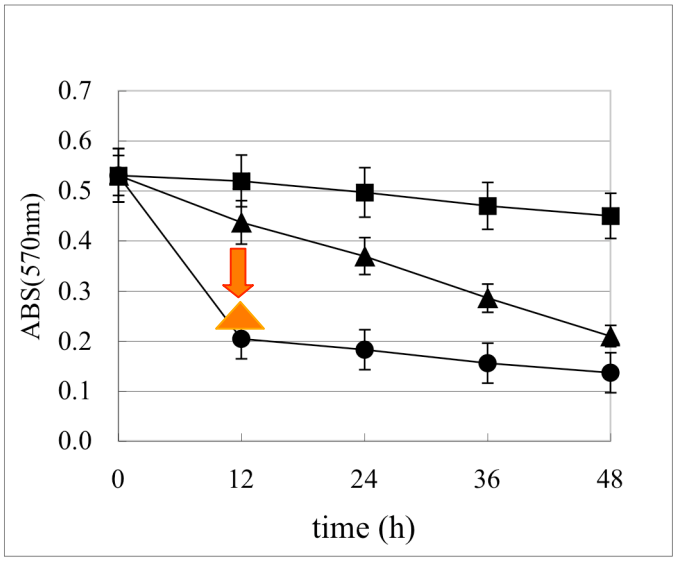


Fig. S1. HPLC patterns of the EtOAc-extract from VioABDE.
Left: MeOH:H_2O=45:55; Right: MeOH: 20 mM NH_3-buffer=45:55.

Details for the experiments of Fig. 2 (main text), Fig. S2 and Fig. S3.

The cell-free extracts (60 ml each) were separately obtained from 300-ml culture of VioA, B, C, D and E. For each of the experiments, 0.5 ml of each of the cell-free extracts was used. When NADPH is required, 2.25 mg was added to the incubation mixture. The EtOAc-extract (usually 4 ml) contains **8** or **9** was quickly concentrated to a small volume (usually ca 20 µl; first: by rotary evaporator, 2nd: by passing a stream of N_2 gas), which was added to VioC or VioCD, then further incubated at 25°C for 12 h on a rotary shaker at 120 rpm, yielding bluish-purple violacein **1**. To the incubation mixtures, EtOAc was added in order to extract the blue pigments thus produced. The EtOAc solution was evaporated to dryness. The production amounts of **1** were monitored at 570 nm after dissolving the residues in MeOH (5 ml).

3. Stabilities of intermediate 8.



O_2 was bubbled into the EtOAC-extract at the initial time of 0 h, and continued for 12 h.

Fig. S2. Stability of intermediate **X** (**8**) in the solution of EtOAc, when stood at -20°C (■) and at 25°C (▲). The symbol (●) shows the stability when the EtOAc solvent was dried. The stabilities were determined by estimating **1** produced by adding VioC in the presence of NADPH. The symbol (⇨) indicates the stability of intermediate **X** after bubbling O_2 into the EtOAc solution. This experiment shows that the intermediate **8** is very labile to O_2. The stability of **8** in the EtOAc solution is higher than that of culturing in the LB-medium (compare Fig.S2 with Fig. 2 of the main text). This is due to the higher concentration of the dissolved O_2 in the culture medium, because the rotary shaking is being carried out in the experiment of Fig. 2 of the main text.

4. Electronic spectra against times stood at room temperatures.

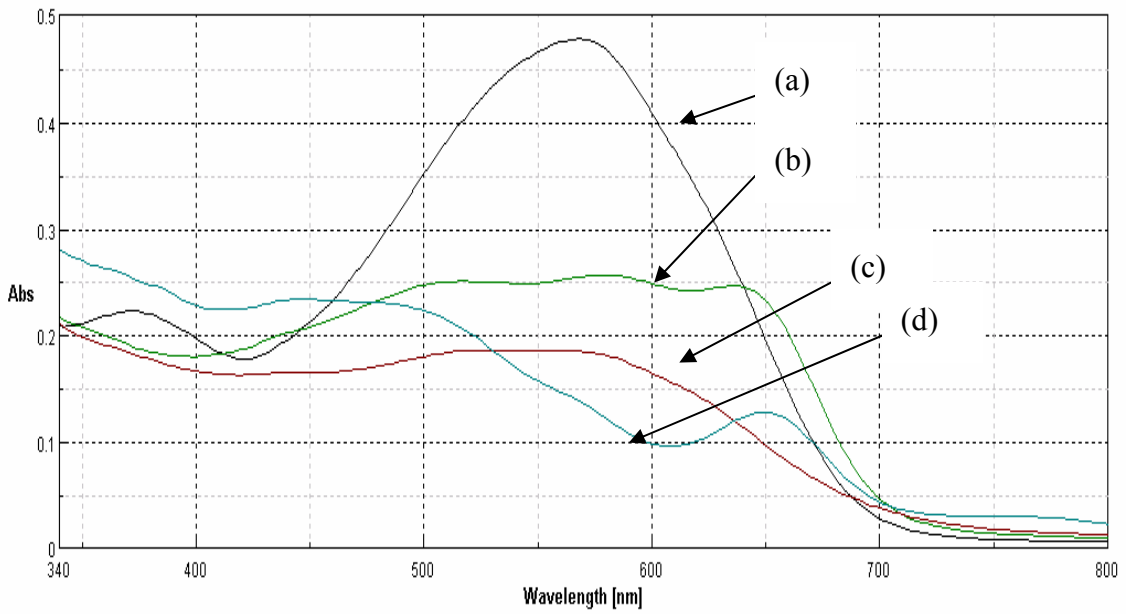


Fig. S3. Visible spectra of the pigments produced by adding VioC into the EtOAc-extract containing **8**. The produced pigments were **1**,**2**,**6** and **7** (see Fig. 3 of the main text). The pigments were extracted with EtOAc from the incubation mixture, and then dissolved in MeOH. Note that **1**,**2**,**6** and **7** are stable compounds, difffering from intermdiates **8** and **9**.

(a): The EtOAc solution containing **8** was stood for 12 h at 25°C, followed by addition of VioC in the presence of NADPH to give violacein **1**. Then the pigments thus produced were extracted with EtOAc and the spectrum was measured in MeOH. This spectrum (the shape and λ_{max}) is identical to that of authentic **1**.
(b): To the EtOAc-extract containing **8**, O_2 gas was bubbled for 12 h at 25°C, followed by additon of VioC in the presence of NADPH. Then the pigments thus produced were extracted with EtOAc and the spectrum was measured in MeOH.
(c): The EtOAc solution containing **8** was dried and stood for 12 h at 25°C (the exposure to air), followed by additon of VioC in the presence of NADPH. Then the pigments thus produced were extracted with EtOAc and the spectrum was measured in MeOH.
(d): The EtOAc solution containing **8** was dried and stood for 48 h at 25°C(the exposure to air), followed by additon of VioC in the presence of NADPH. Then the pigments thus produced were extracted with EtOAc and the spectrum was measured in MeOH. (c) is different from (d) only regarding the exposure time to air: (c), 12 h; (d), 48h.

Note that the visible spectra of (b), (c) and (d) are significantly different from that of violacein pigment **1** (a). TLC analysis showed that large amounts of **6** and **7** were produced in the experiments of (b), (c) and (d) (see Fig. 2 of the main text).

5. Production of violacein 1 uner the following conditions:

VioABE→EtOAc-extract→VioCD+NADPH.

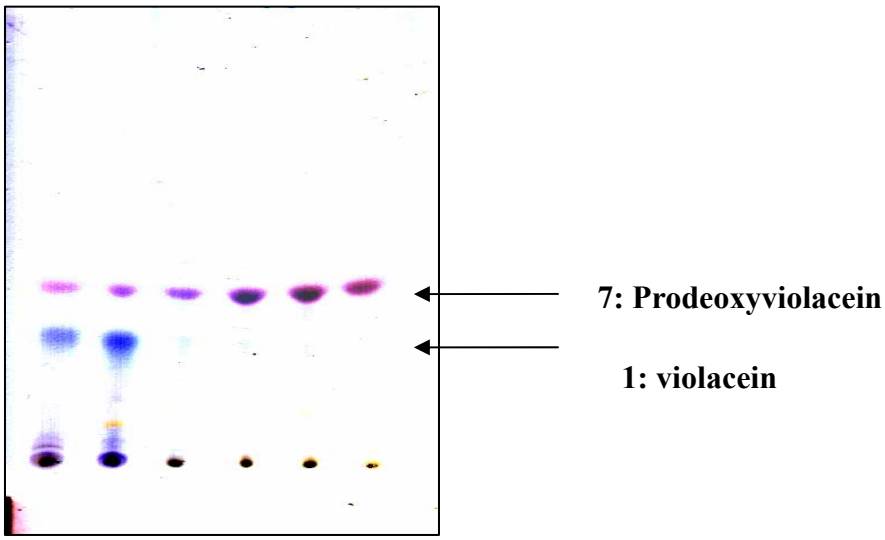
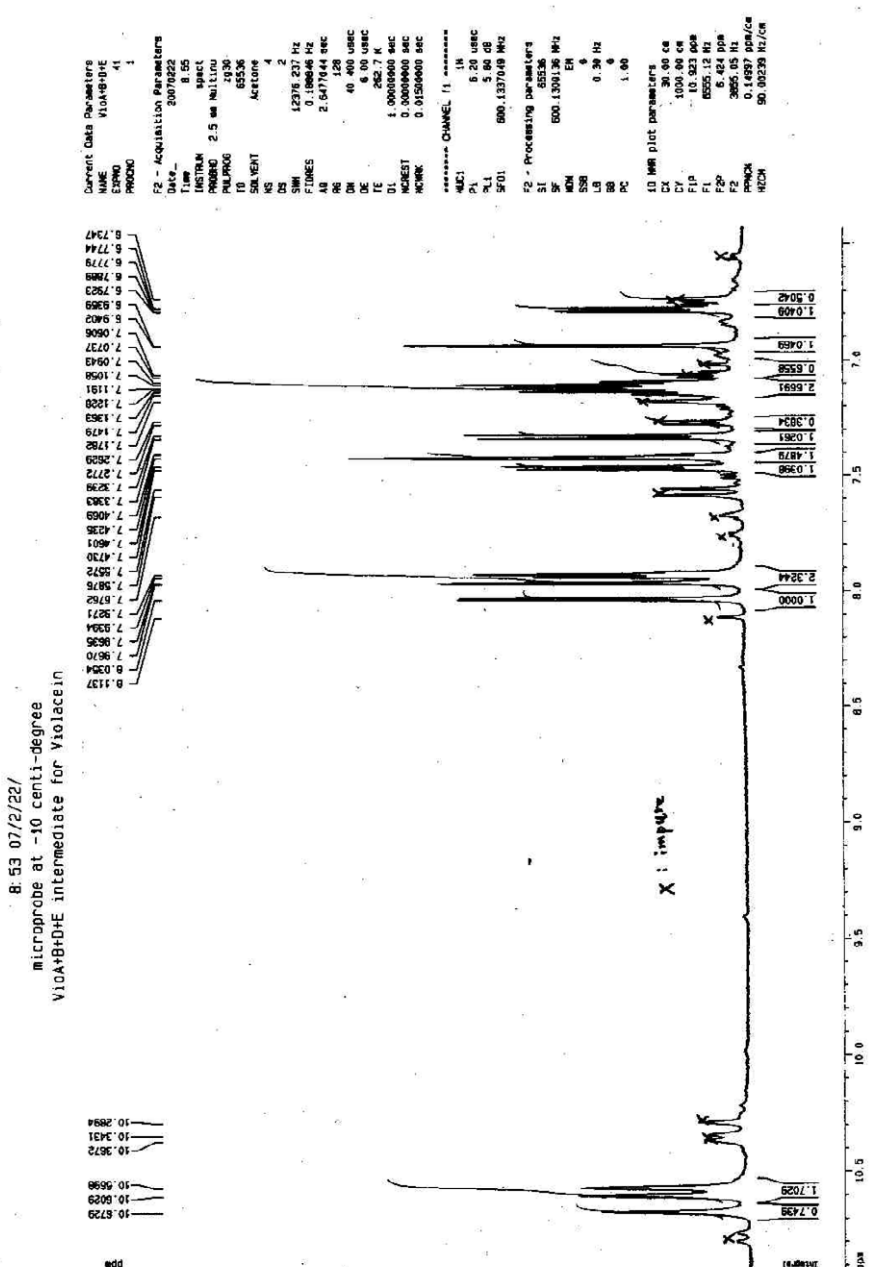


Fig. S4. SiO_2-TLC developed four times with MeOH:$CHCl_3$ (9: 0.5). Trp was incubated with VioABE for different incubation times (0.5-24 h). The EtOAc-extract obtained from each of incubation times was added to the mixture of VioC in the presence of NADPH, giving rise to violacein **1**. However, the production of **1** was observed only from incubating for 0.5-1 h with VioABE. Longer incubation (> 3h) did not afford **1**, but the production amount of **7**, in turn, was relatively increased with the longer incubation times (>5 h). In this experiment, no production of proviolacein **6** was found, indicating that prodexyviolacein **7** is not converted into **6** by VioD.

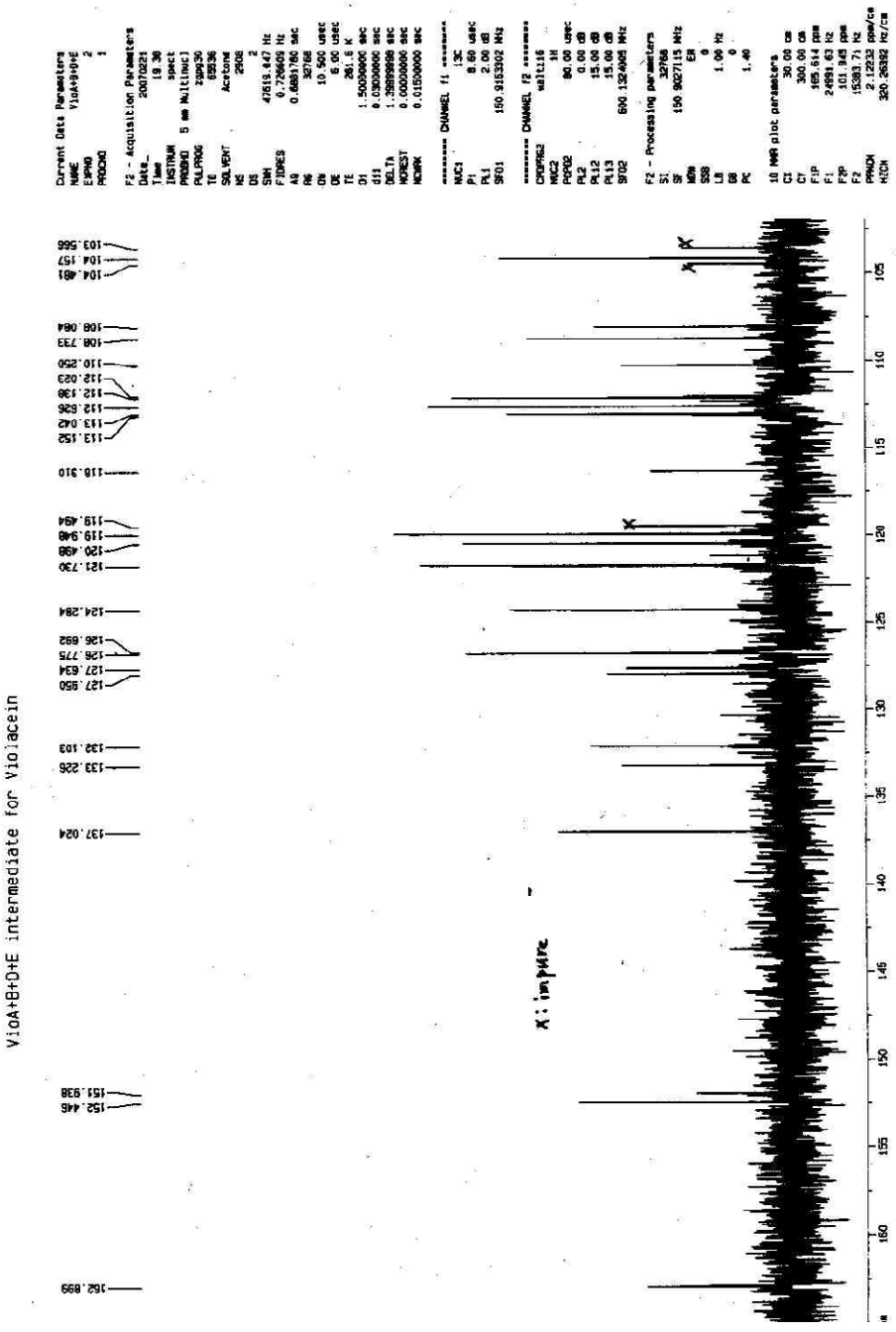
6. NMR spectra of 8 measured at -10°C in acetone d_6 (600 MHz, Bruker DMX600)

^{1}H NMR

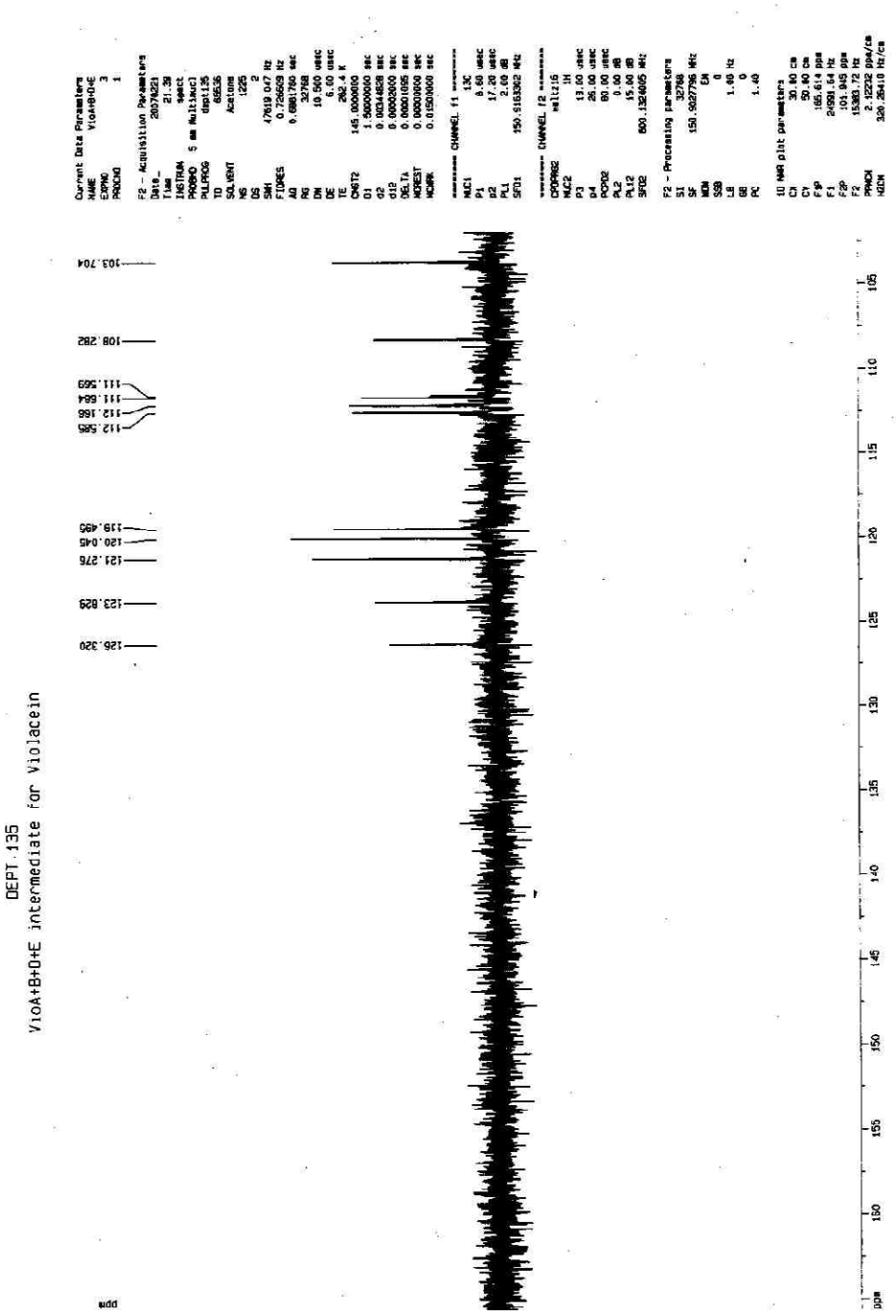


Remark: In the ^{1}H and ^{13}C NMR spectra, some peaks from the impurity were observed. This is due to instability of **8**, but in case of the methyl ester of **8**, no decomposition occurred, thus the NMR signals from the impurity were minimum.

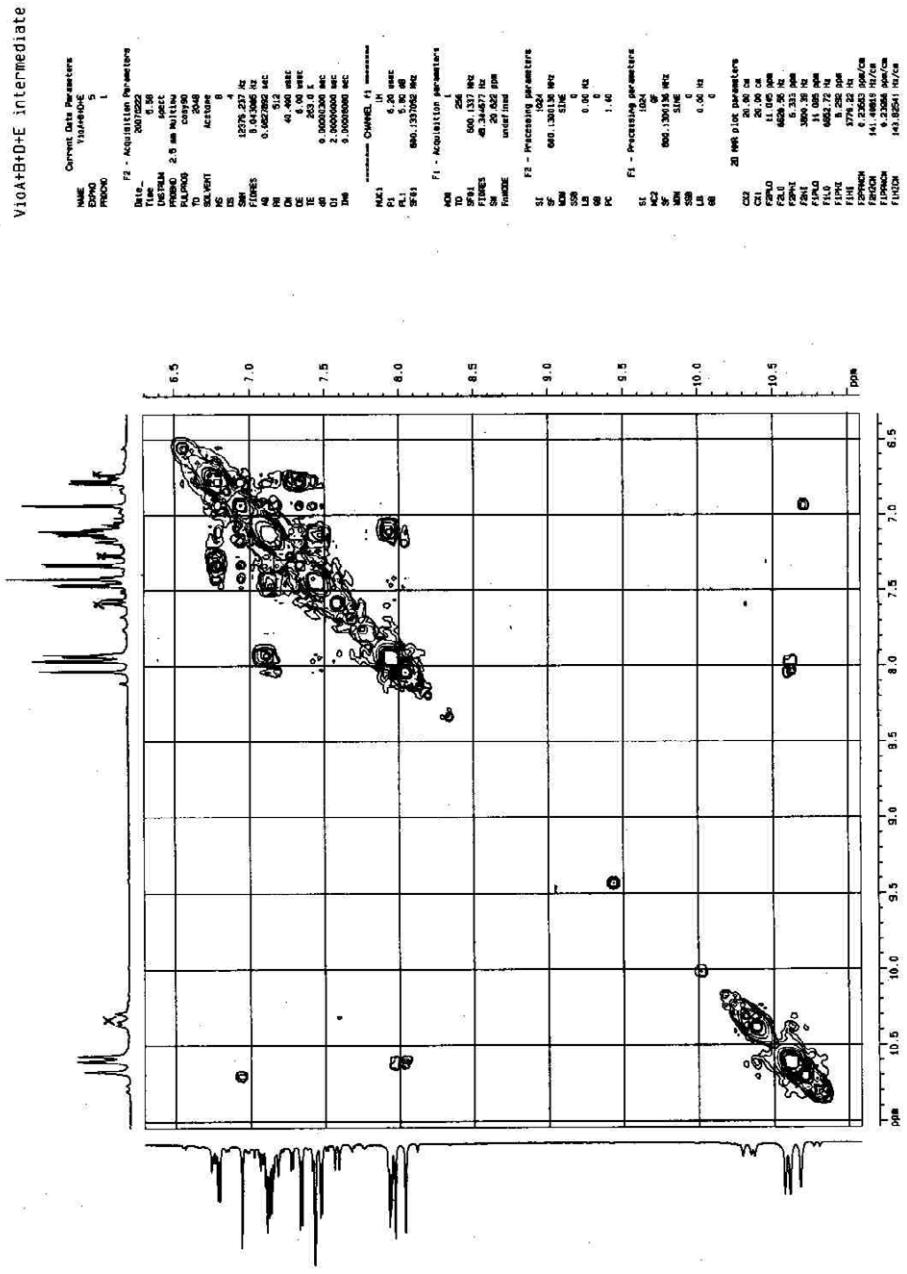
^{13}C NMR



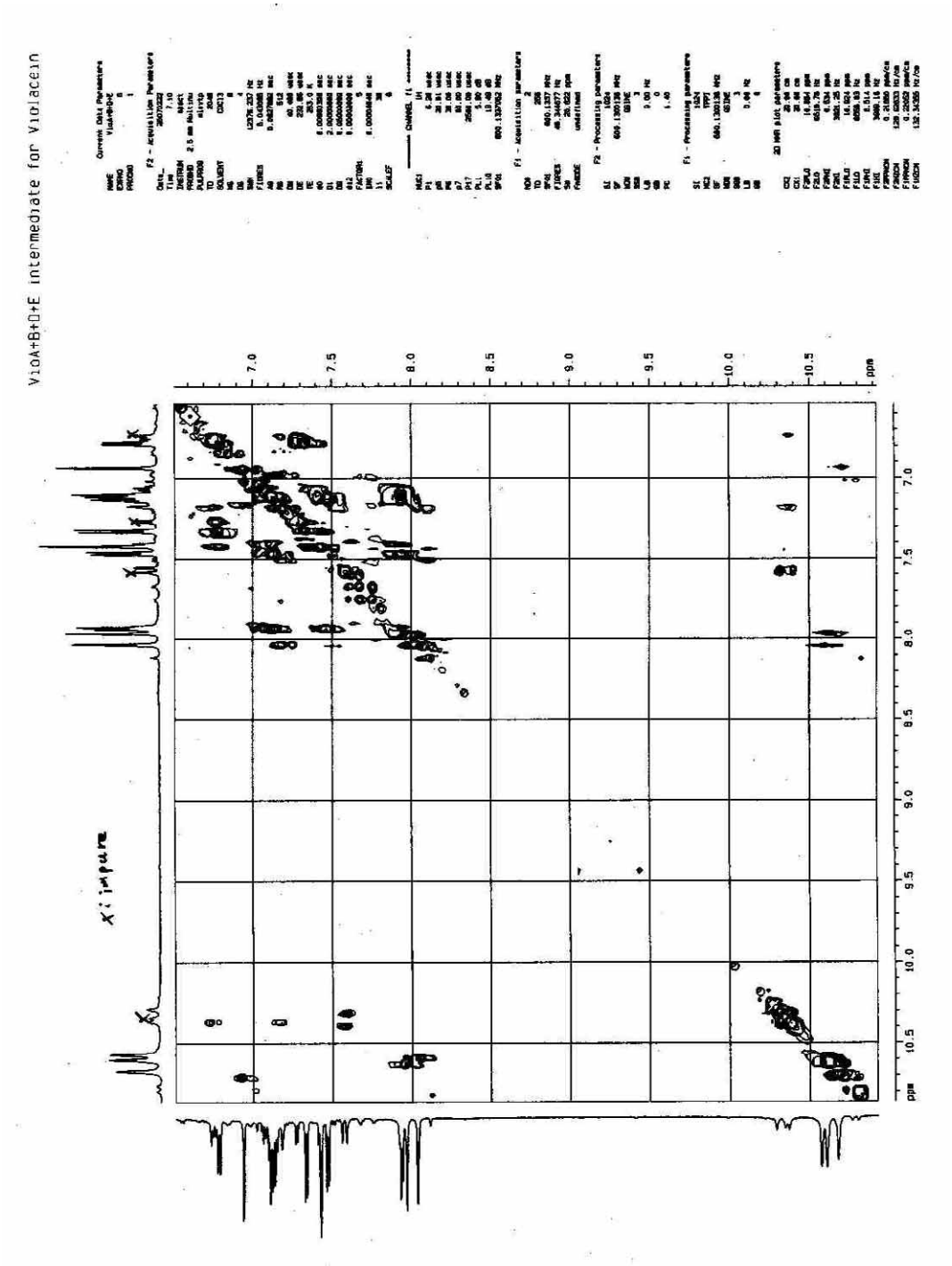
DEPT 135



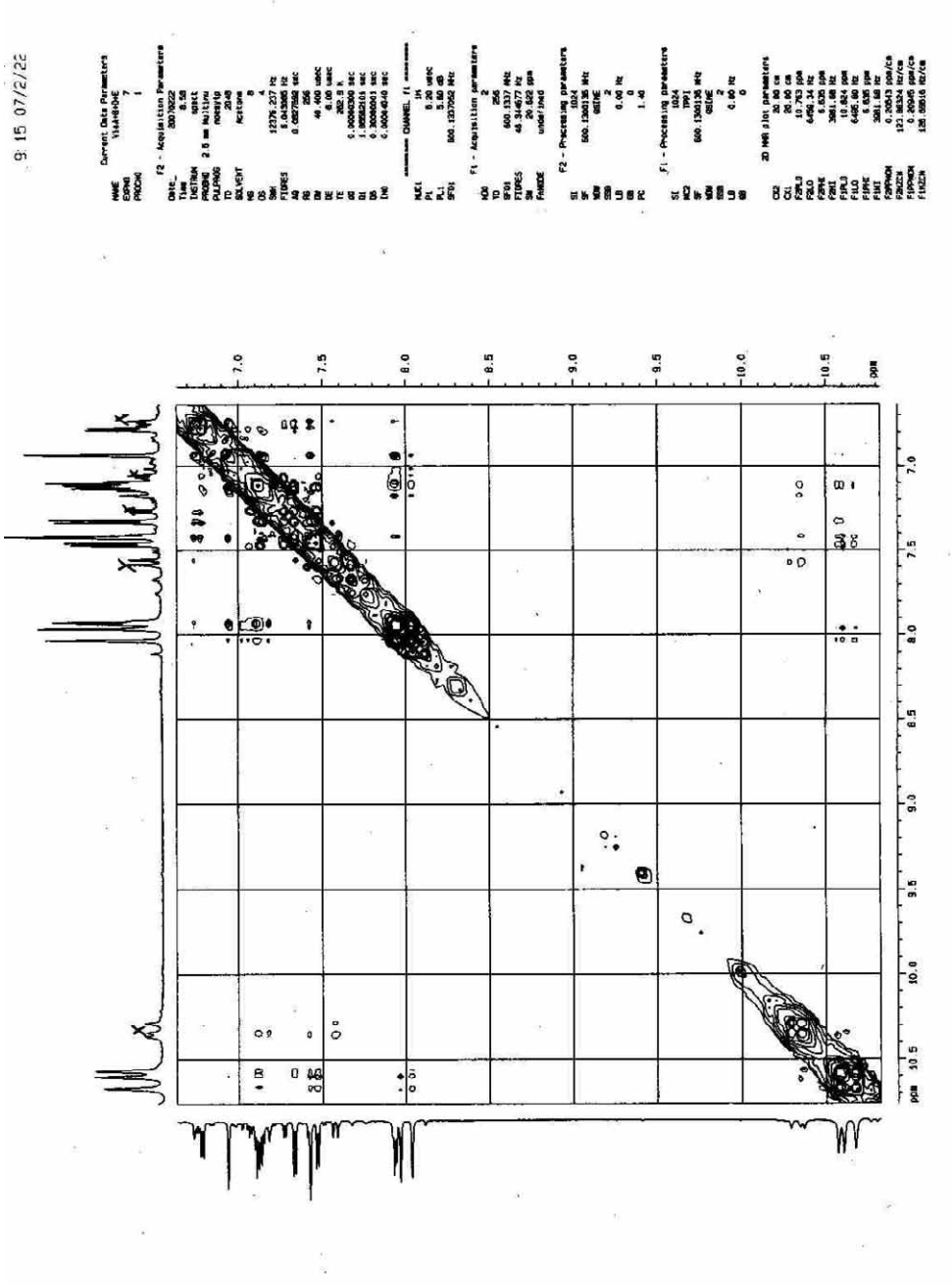
COSY 90



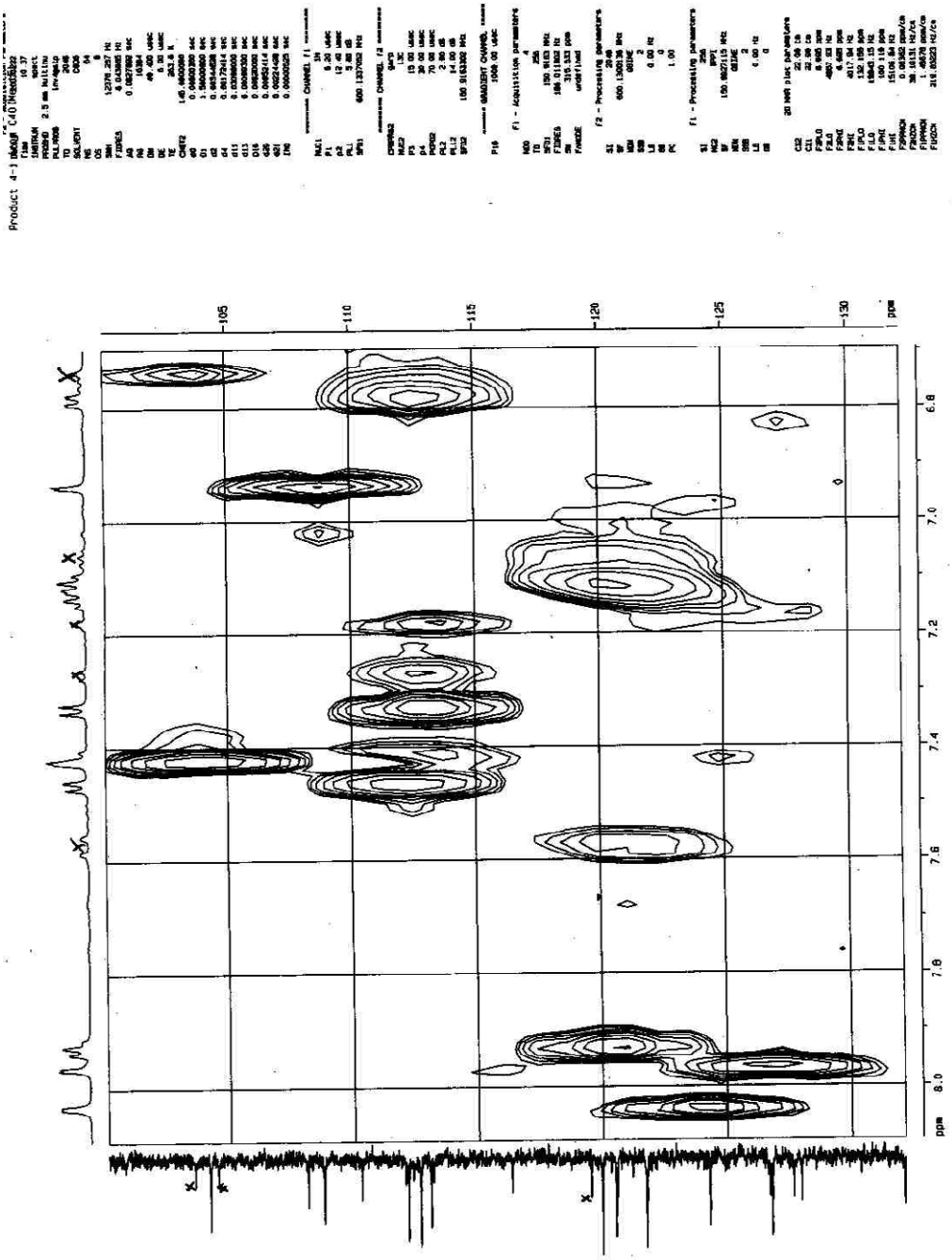
HOHAHA


VioA+B+D+E intermediate for Violacein
X : impure
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
ppm

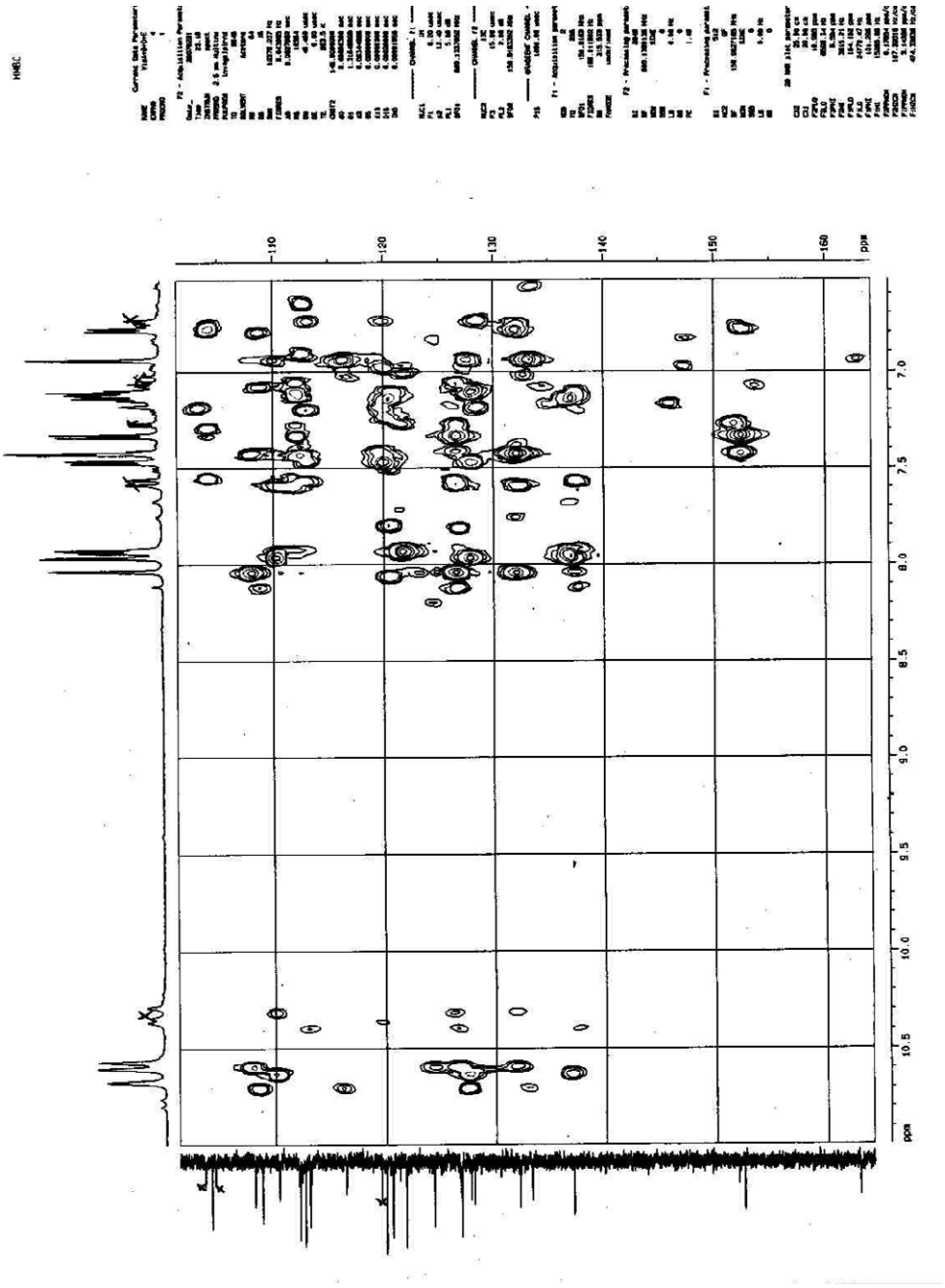
NOESY



HMQC



HMBC



7. NMR assignments of 8 measured at -10·°C in acetone-d_6.

Vio (A+B+D+E) + Trp

↓ Incubation for 1 hr

EtOAc extract

↓

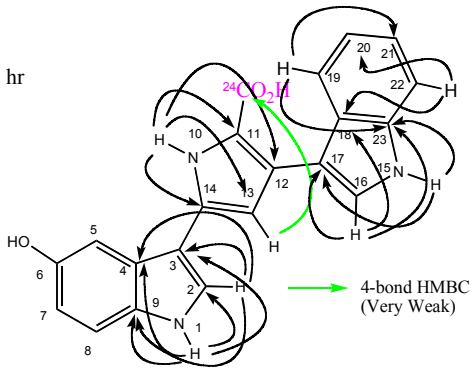
SiO_2 column eluted with EtOAc

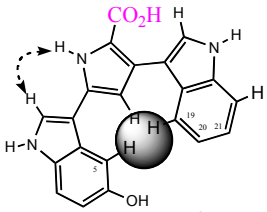
↓

Reversed Phase HPLC

↓

NMR in $(CD_3)_2CO$ at -10°C





HMBCs from N*H* was clearly found at -10°C

Measured at -10°C in acetone-d_6 with 600 MHz δ_H and δ_C were given in relative to the solvent peaks of 2.04 ppm and 29.8 ppm

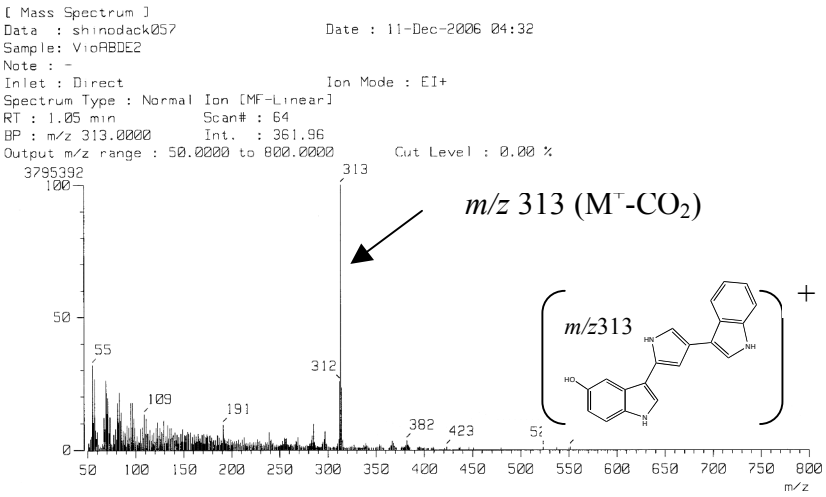
No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C
1	10.57 (bs, NH)	—	6	—	152.4	11	—	127.6[a]	16	7.95(d, 2.1Hz)	126.8	21	7.14(t,7.5Hz)	121.7
2	8.03(d, 2.0Hz)	124.3	7	6.77(dd, 2.0,8.6Hz)	112.6	12	—	116.3[a]	17	—	110.2	22	7.47(d, 7.5Hz)	112.1
3	—	108.1	8	7.33(d,8.6Hz)	113.0	13	6.94(d, 2.5Hz)	108.7	18	—	127.9	23	—	137.0
4	—	126.7	9	—	132.1	14	—	133.2	19	7.93(d,7.5Hz)	120.5	24	—	162.9
5	7.42(d, 2.2Hz)	104.1	10	10.67(bs, NH)	—	15	10.60(bs, NH)	—	20	7.11(t, 7.5Hz)	119.9			

*a:*The chemical shifts of C11, C12 may be exchangeable

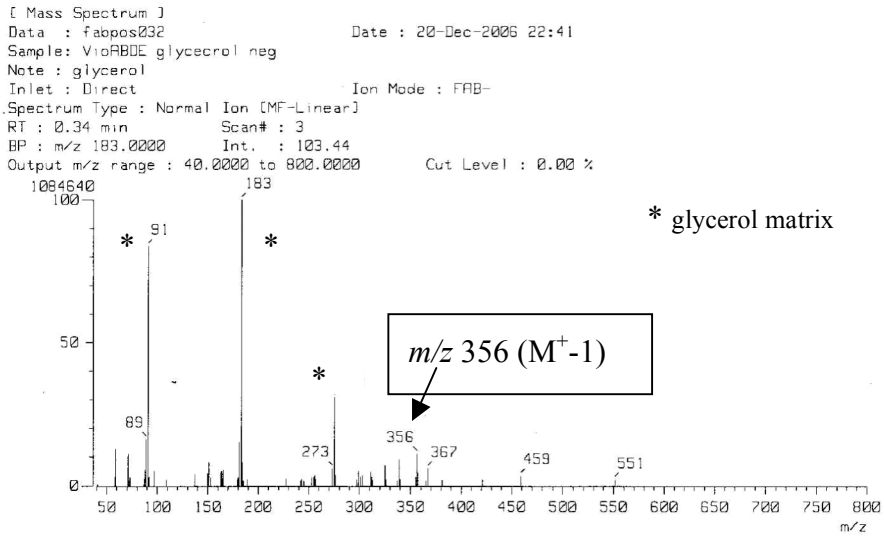
NMR spectra were recorded in acetone d_6 on a Bruker DMX 600 spectrometer, the chemical shifts being relative to the solvent peak δ_H 2.04 and δ_C29.8 ppm as the internal reference for ^{1}H- and ^{13}C NMR spectra, respectively.

8 EI- and FAB-MS of 8.

8-1. EI-LRMS (70 eV)

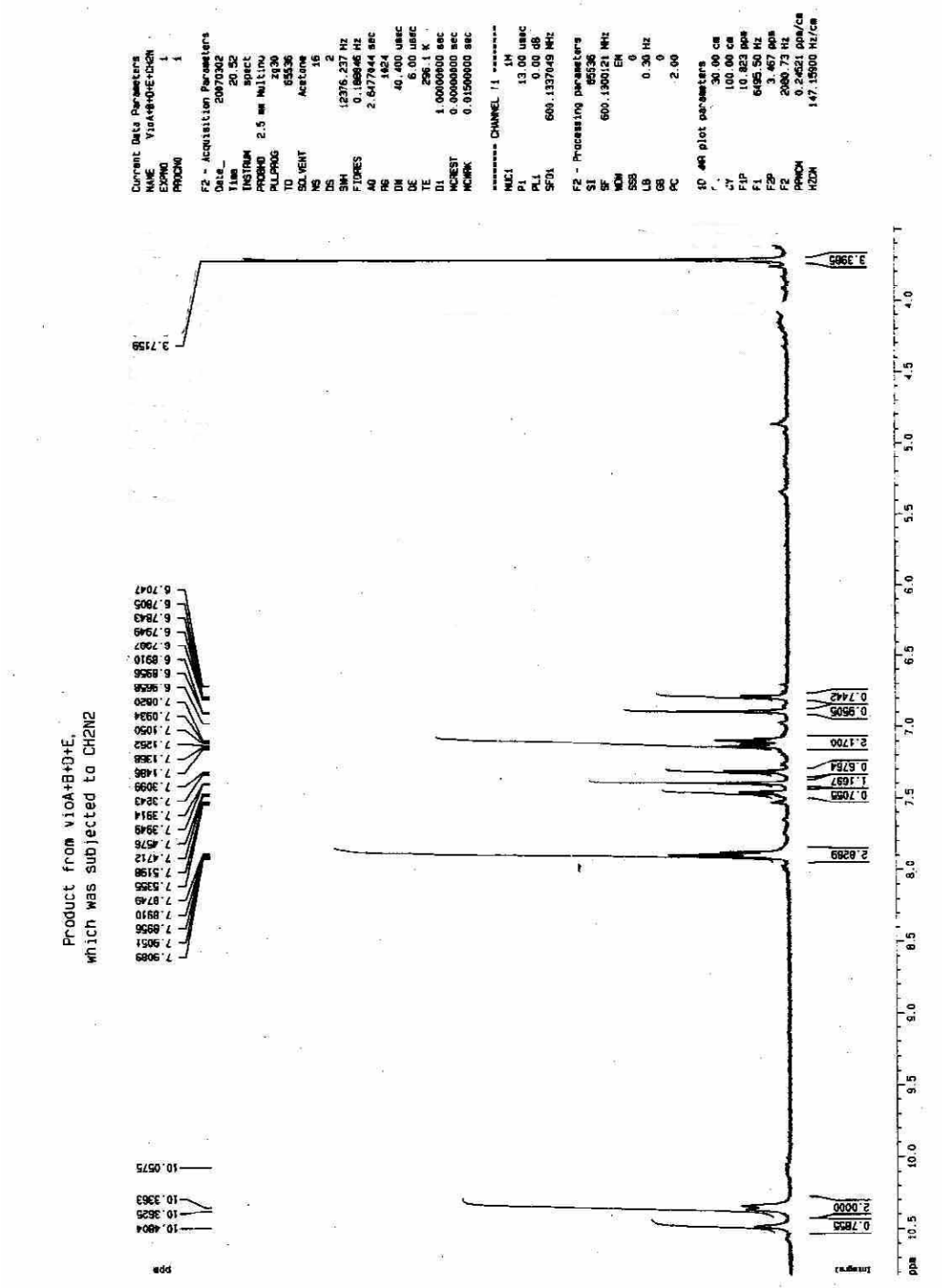


8-2. FAB-LRMS (negative, glycerol matrix)

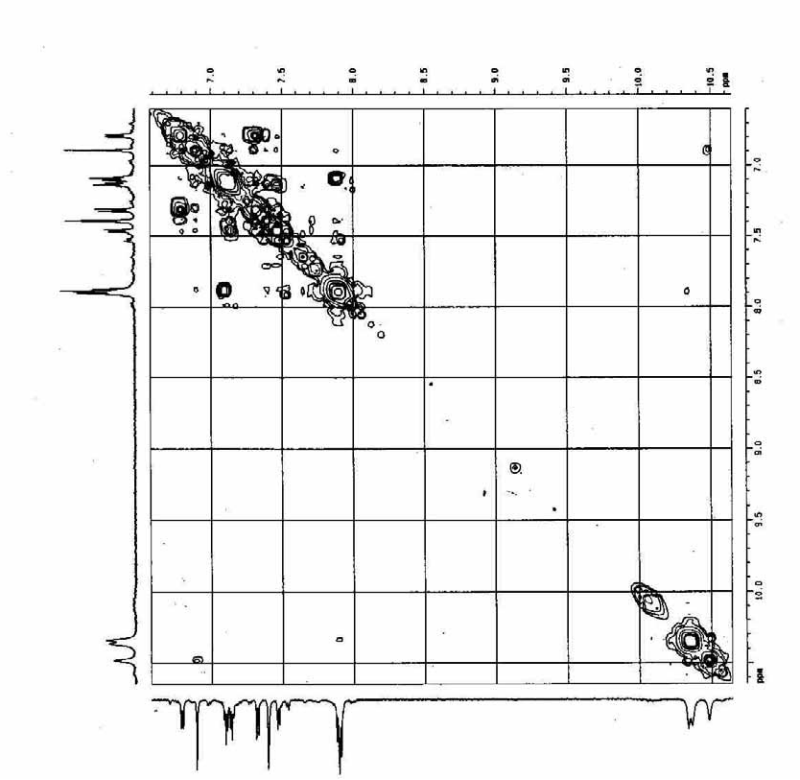


9. NMR spectra of the methyl ester of 8 measured at 25°C in acetone d_6

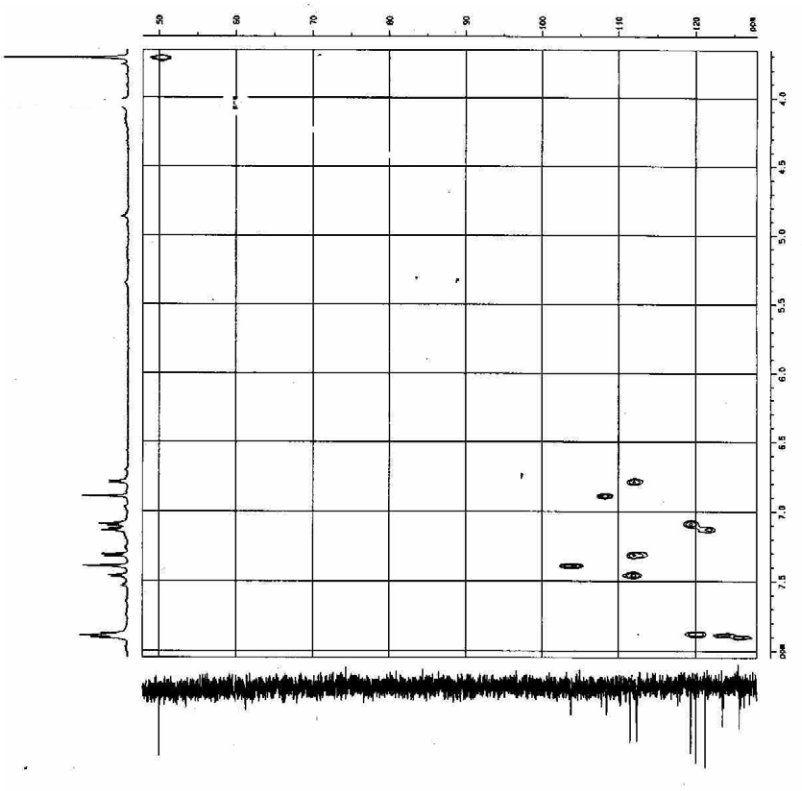
^{1}H-NMR (600 MHz)



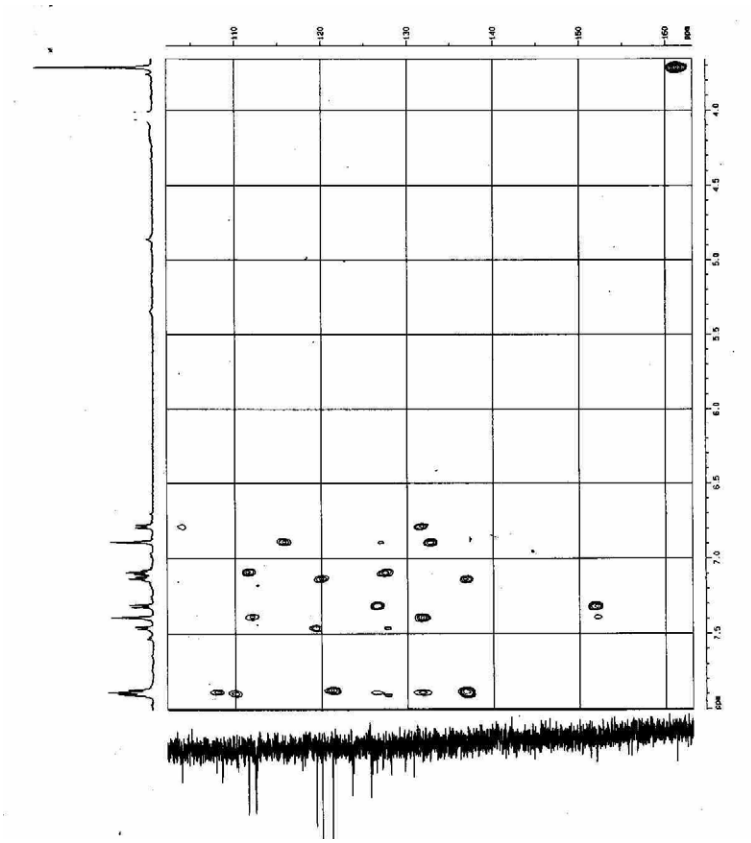
COSY 90



HMQC

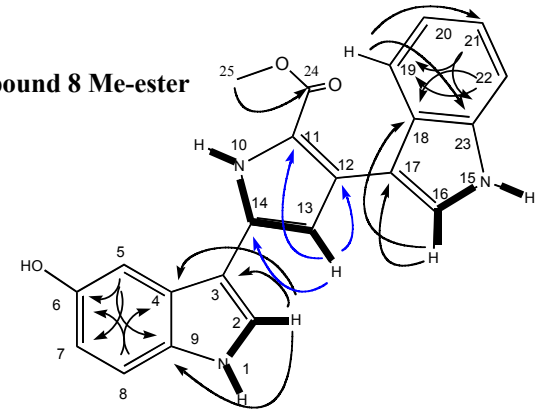


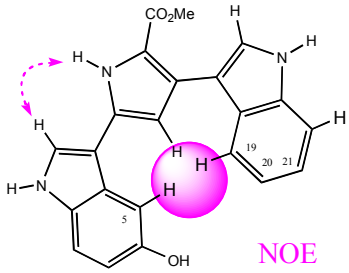
HMBC



10. NMR assignments of methyl ester of 8 in acetone-d_6.

Compound 8 Me-ester





Measured at -10°C in acetone-d_6 with 600 MHz δ_H and δ_C were given in relative to the solvent peaks of 2.04 ppm and 29.8 ppm

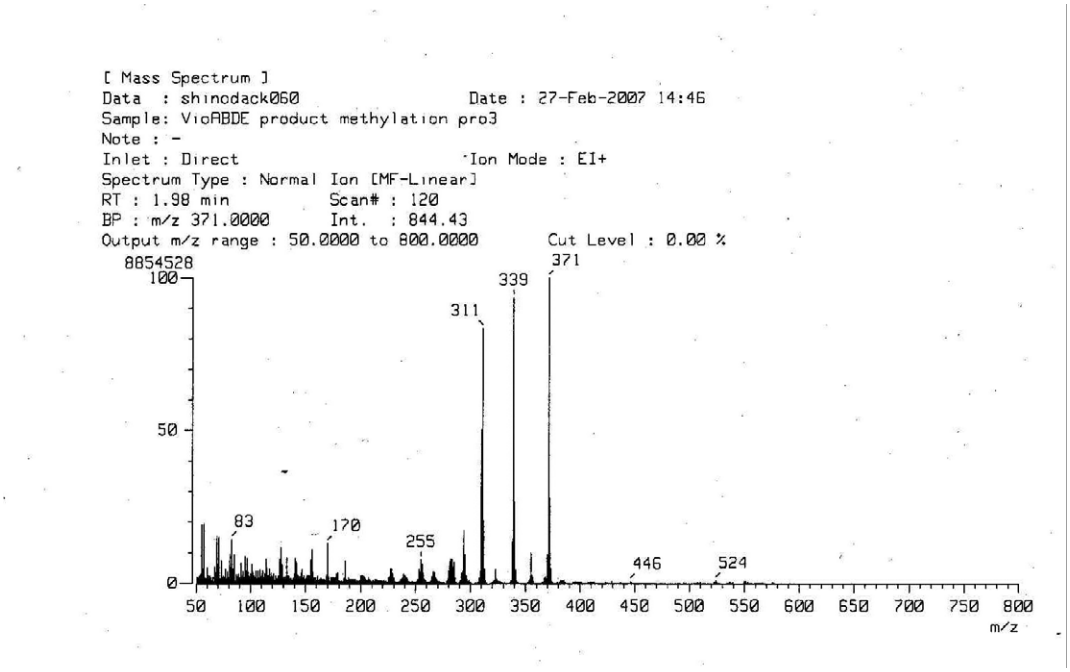
No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C
1	10.34(brs)	—	6	—	151.7	11	—	126.6[a]	16	7.90(d, 2.3Hz)	125.7	21	7.14(bt,8.1Hz)	121.2
2	7.89(d, 2.7Hz)	123.5	7	6.79(dd, 8.6,2.1Hz)	112.3	12	—	116.3[a]	17	—	109.9	22	7.46(d, 8.1Hz)	111.5
3	—	107.8	8	7.31(d, 8.6Hz)	112.4	13	6.89(d, 2.7Hz)	108.5	18	—	127.5	23	—	136.6
4	—	126.4	9	—	131.4	14	—	133.5	19	7.88(d, 8.1Hz)	120.0	24	—	162.9
5	7.39(d, 2.2Hz)	103.8	10	10.48(bs)	—	15	10.36(brs)	—	20	7.09(bt, 8.1Hz)	119.4	25	3.71(3H,s)	50.02

a: The carbon assignments may be exchangeable.

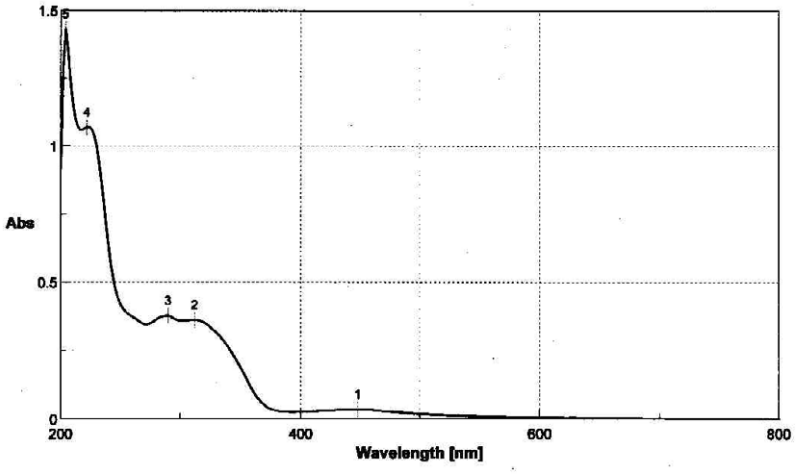
11. EI-LRMS and EI-HRMS of methyl ester of 8.

HREIMS: $C_{22}H_{17}O_3N_2$, Calcd 371.1270, Found 371.1273

EIMS spectra were on a JEOL SX 100 spectrometer under electronic impact at 70 eV (by direct inlet system).



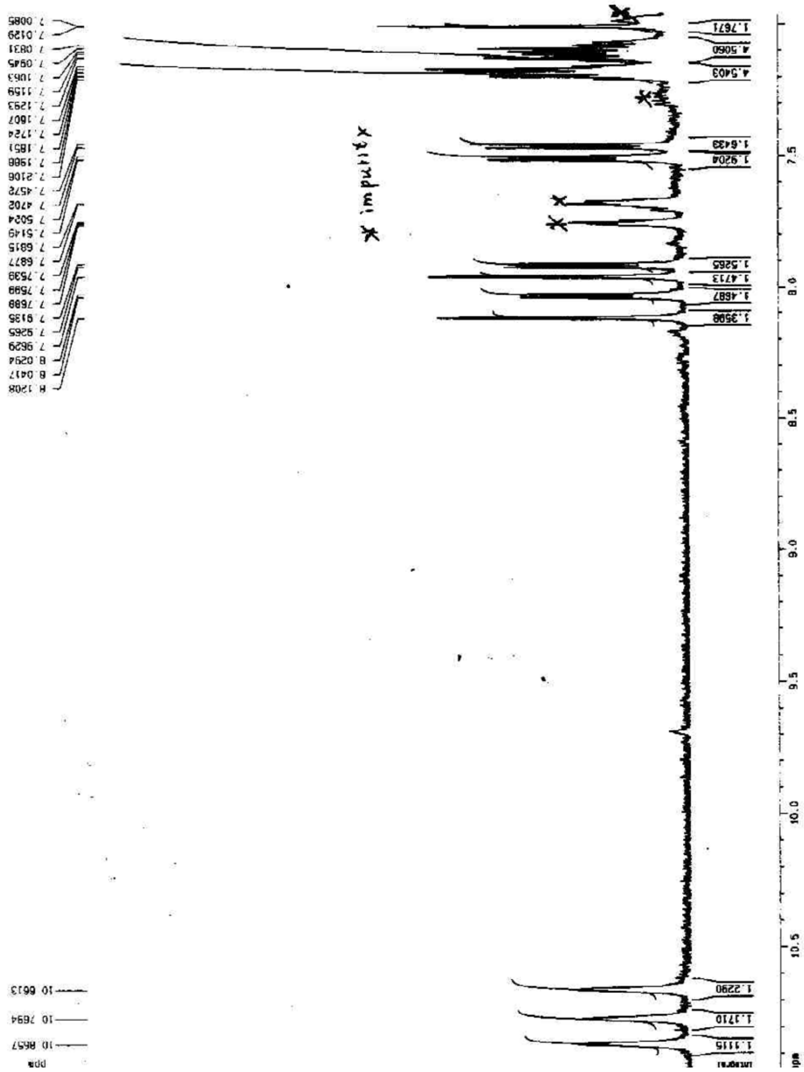
12. Electronic spectrum of methyl ester of 8 in MeOH



Optical pathlength; 1 cm, Concentration 0.330 mg/50 ml MeOH

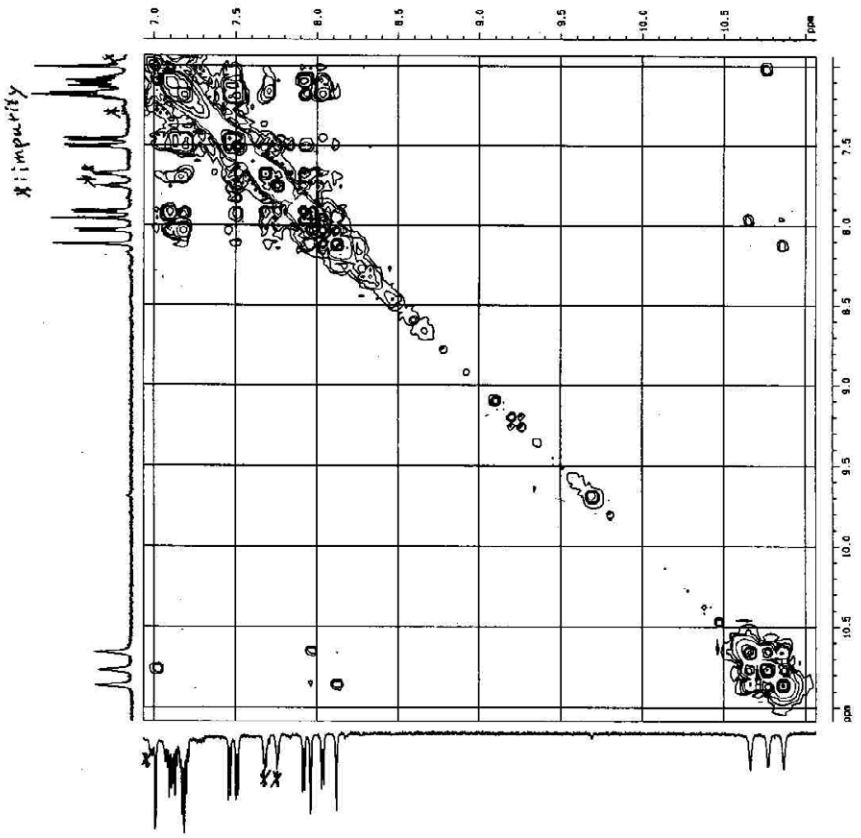
13. NMR spectra of Compound 9 measured at -10°C in acetone d_6

^{1}H-NMR

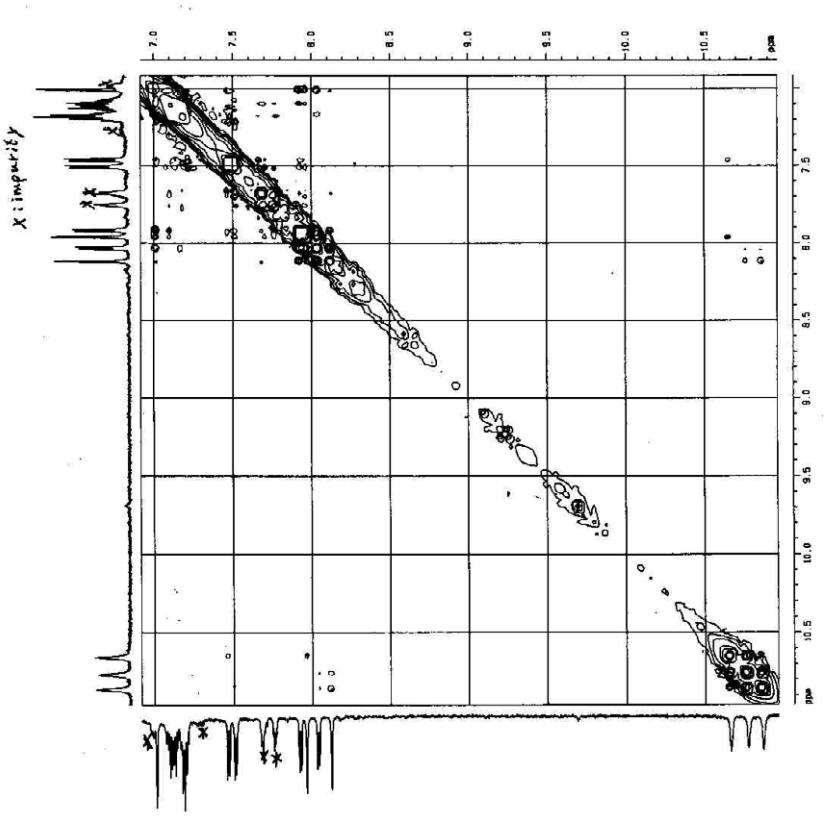


Remark: In the ^{1}H NMR spectrum, some peaks from the impurity were observed. This is due to instability of **9**, but in case of the methyl ester of **9**, no decomposition occurred, thus the NMR signals from the impurity were minimum (S27-S33).

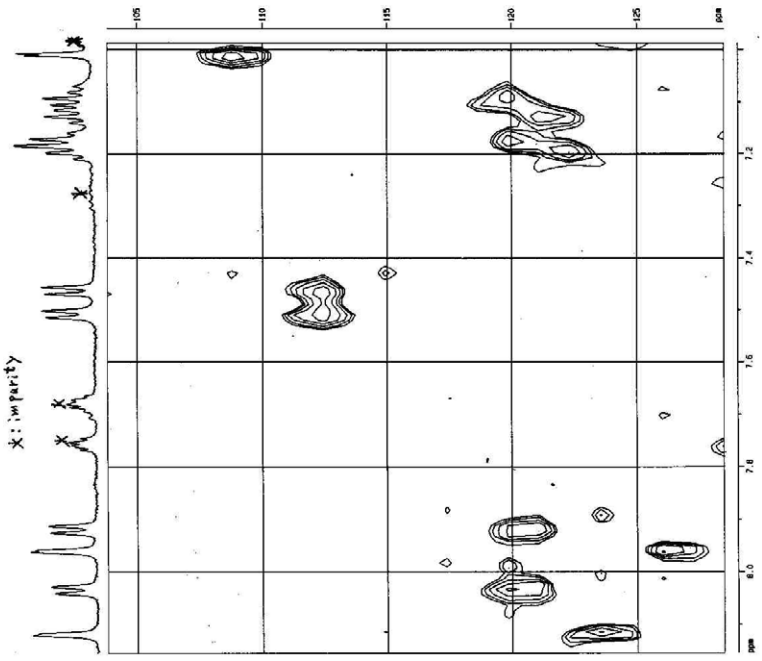
COSY 90



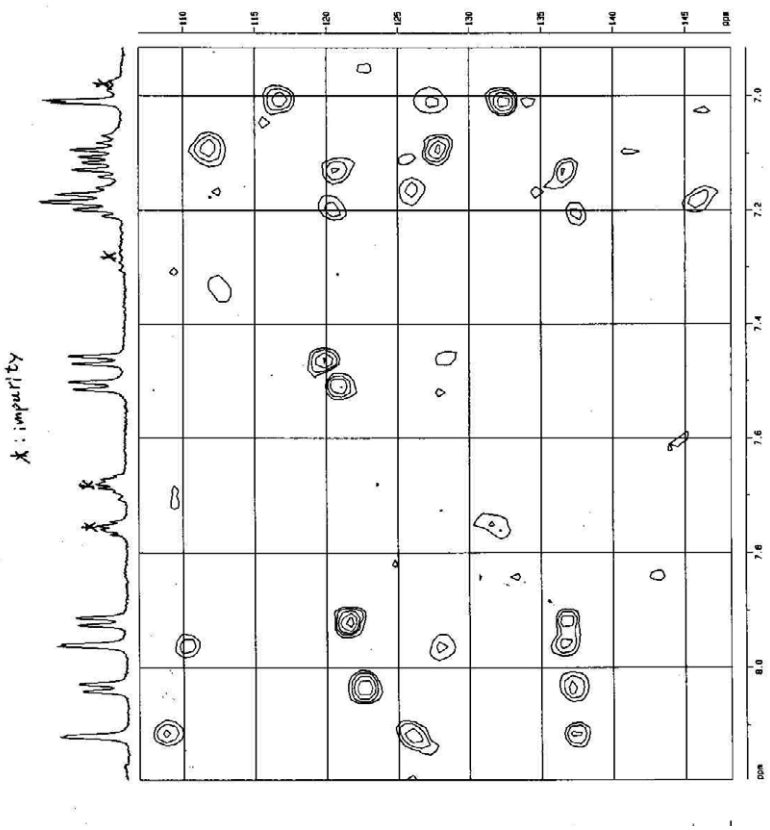
NOESY


7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
ppm
x: impurity

HMQC

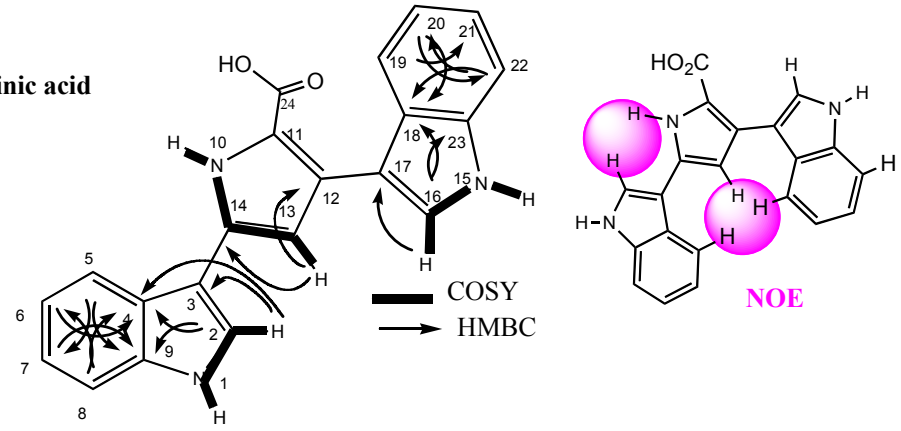

105
110
115
120
125
ppm
x: impurity

HMBC



14. NMR assignments of 9

protodeoxyviolaceinic acid
Compound 9



Measured at -10°C in acetone-d_6 with 600 MHz

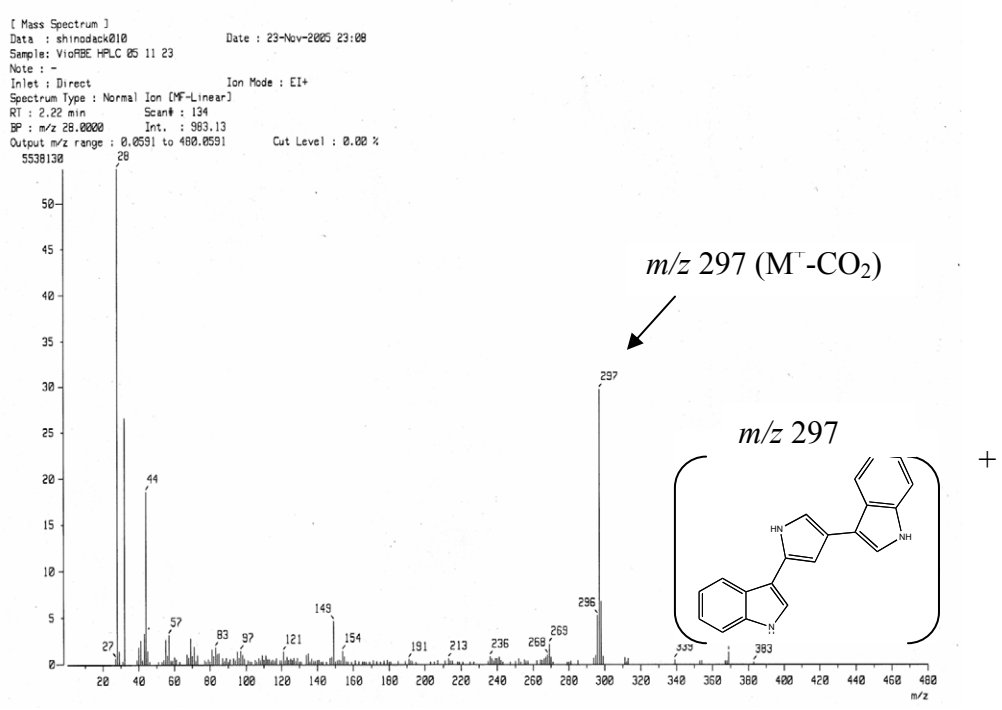
δ_H and δ_C were given in relative to the solvent peaks of 2.04 ppm and 29.8 ppm

No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C
1	10.87(brs)	—	6	7.17(m)	120.8	11	—	127.6[a]	16	7.97(d, 2.0Hz)	126.6	21	7.12(m)	121.9
2	8.12(d, 1.9Hz)	123.9	7	7.20(m)	122.8	12	—	116.8[a]	17	—	110.5	22	7.46 (d, 7.8Hz)	112.3
3	—	109.3	8	7.59(d, 7.5Hz)	112.7	13	6.98(d, 2.8Hz)	109.5	18	—	128.2	23	—	137.4
4	—	126.2	9	—	137.8	14	—	133.2	19	7.92(d, 7.8Hz)	120.7	24	—	160.0
5	8.03(d, 7.4Hz)	120.4	10	10.77(brs)	—	15	10.66(brs)	—	20	7.09 (m)	120.1			

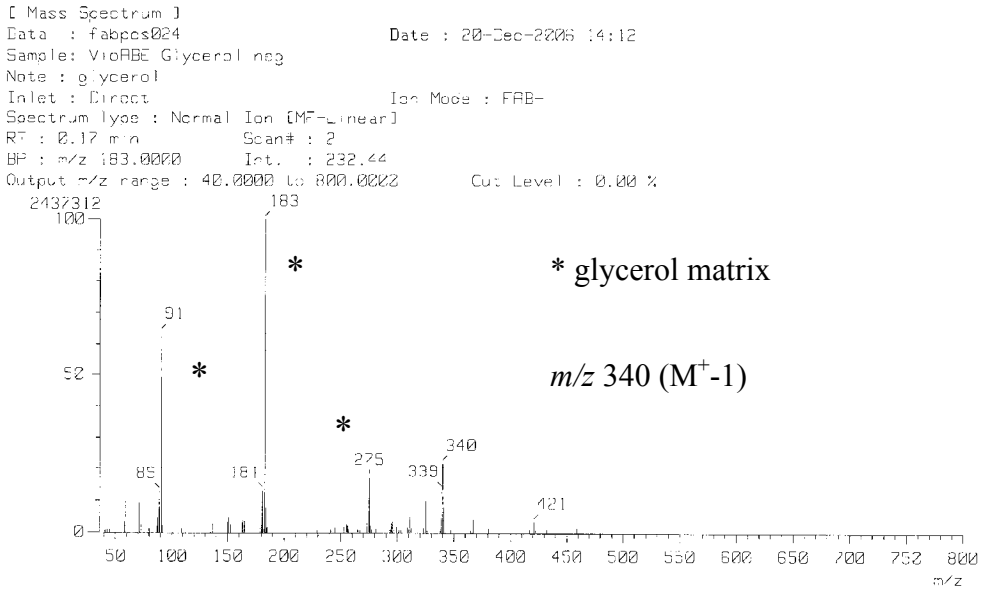
a: The carbon signals of C11 and C12 are exchangeable

15. EI- and FAB-MS of 9

15-1. EI-LRMS

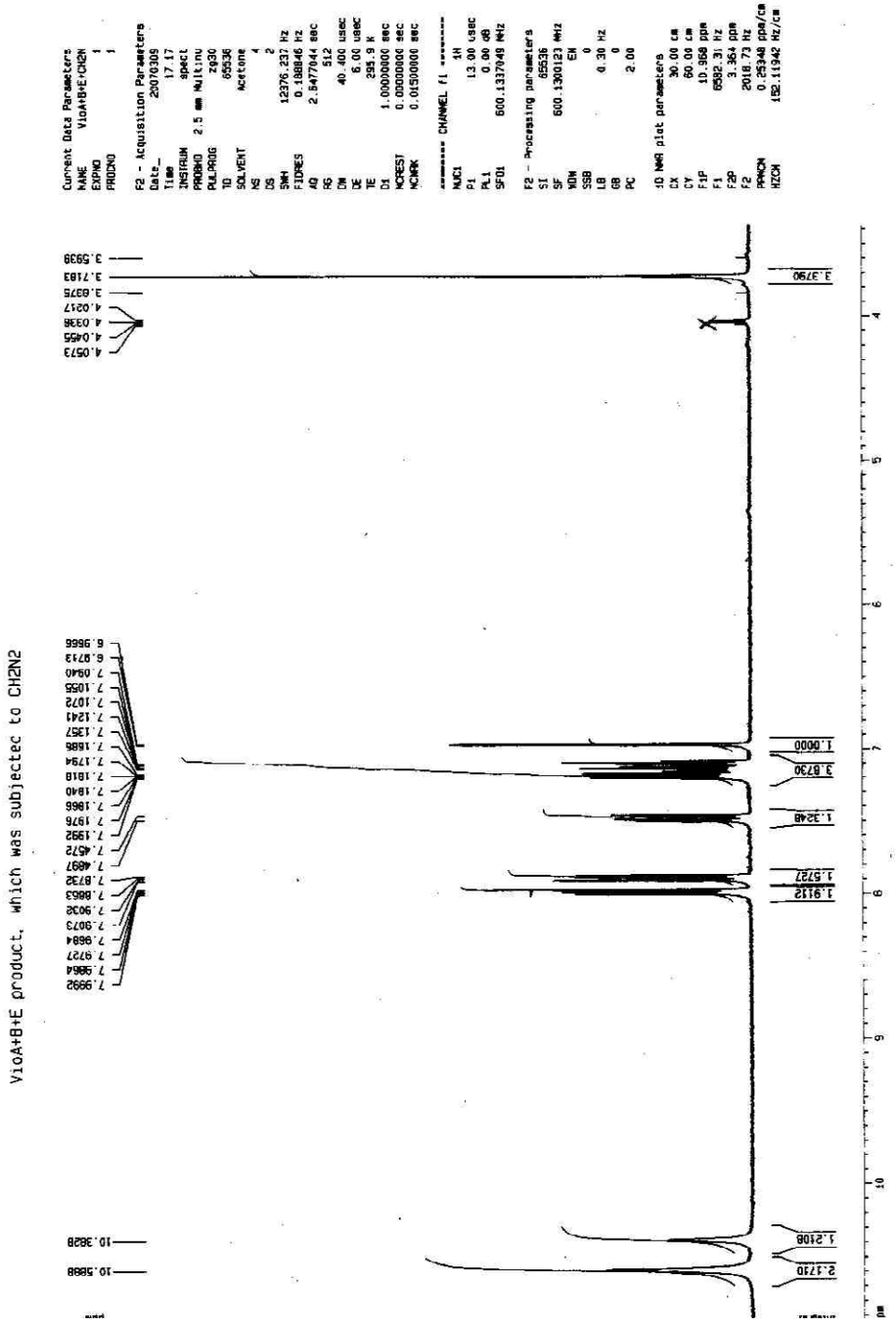


15-2. FAB-LRMS (negative, glycerol matrix)

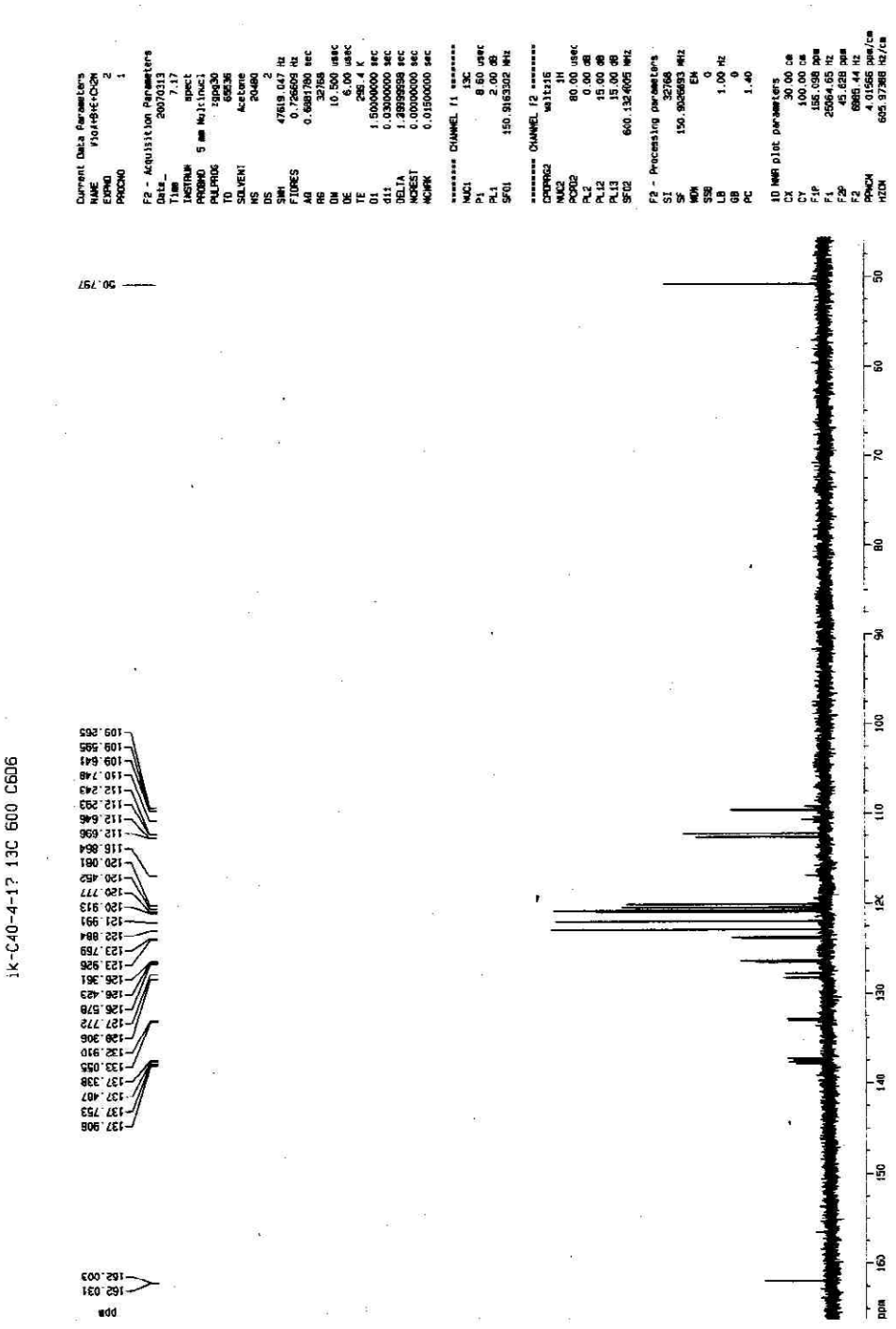


16. NMR Spectra of methyl ester of 9 (DMX 600) in acetone d_6

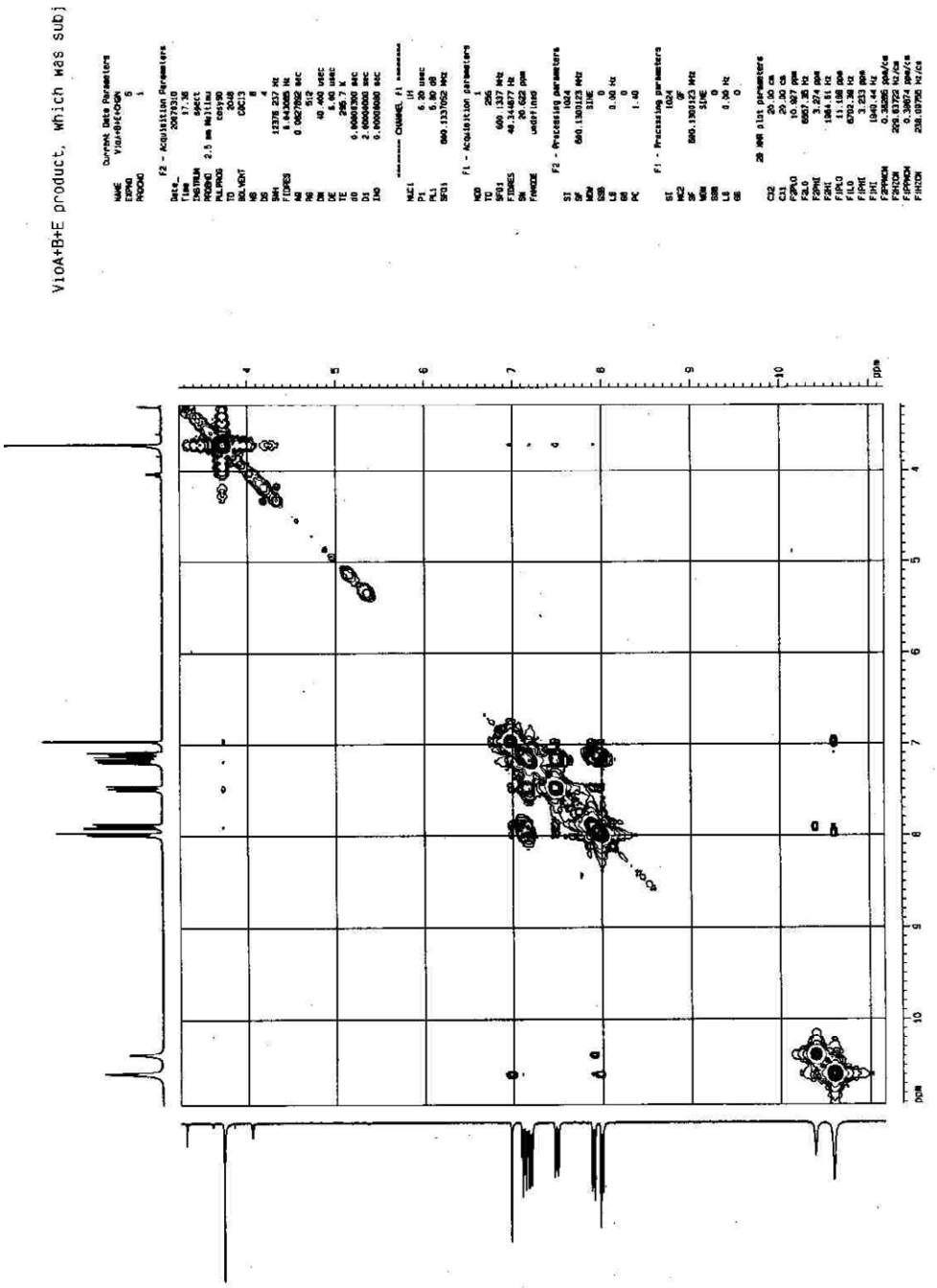
^{1}H NMR



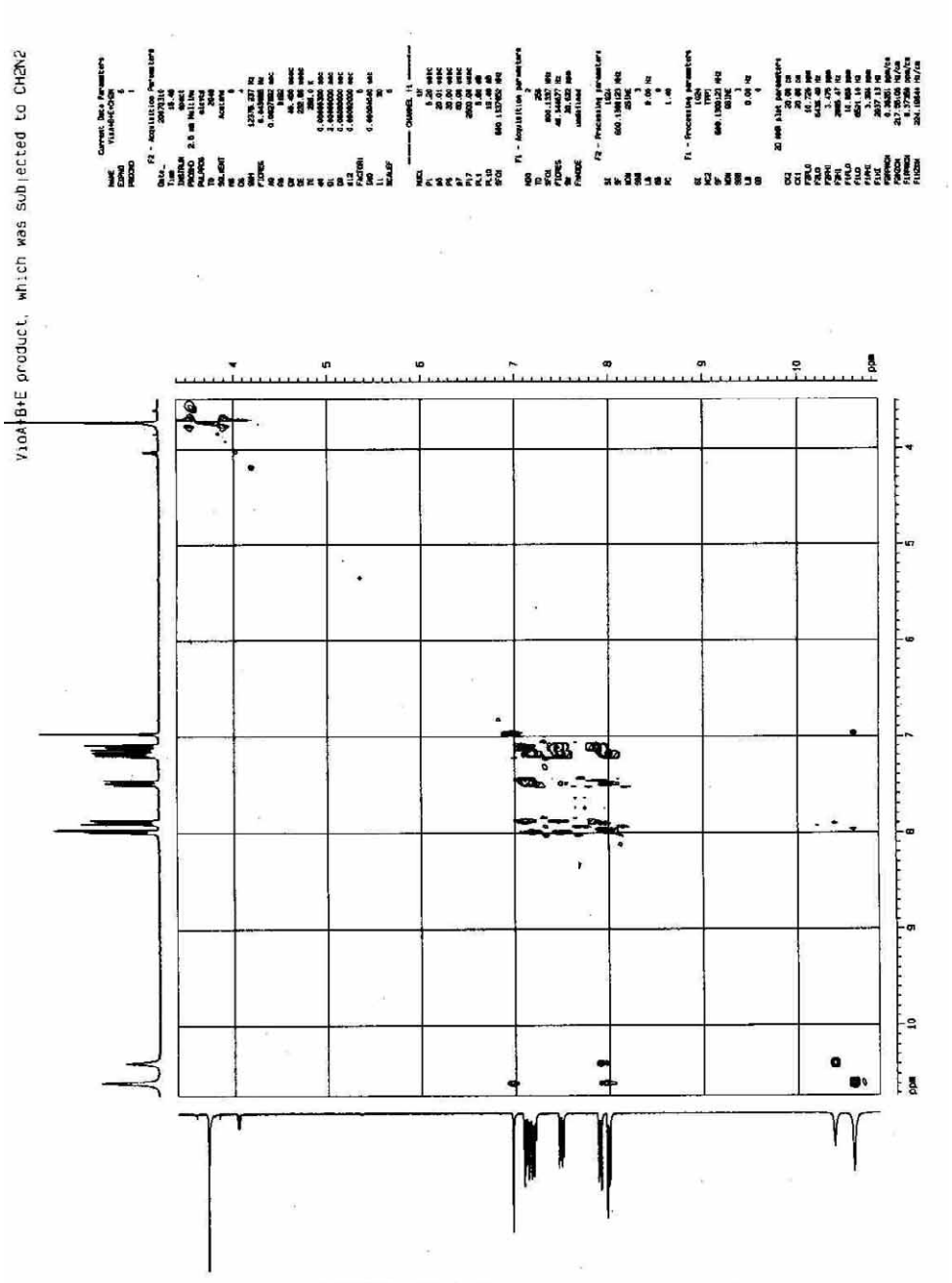
^{13}C-NMR



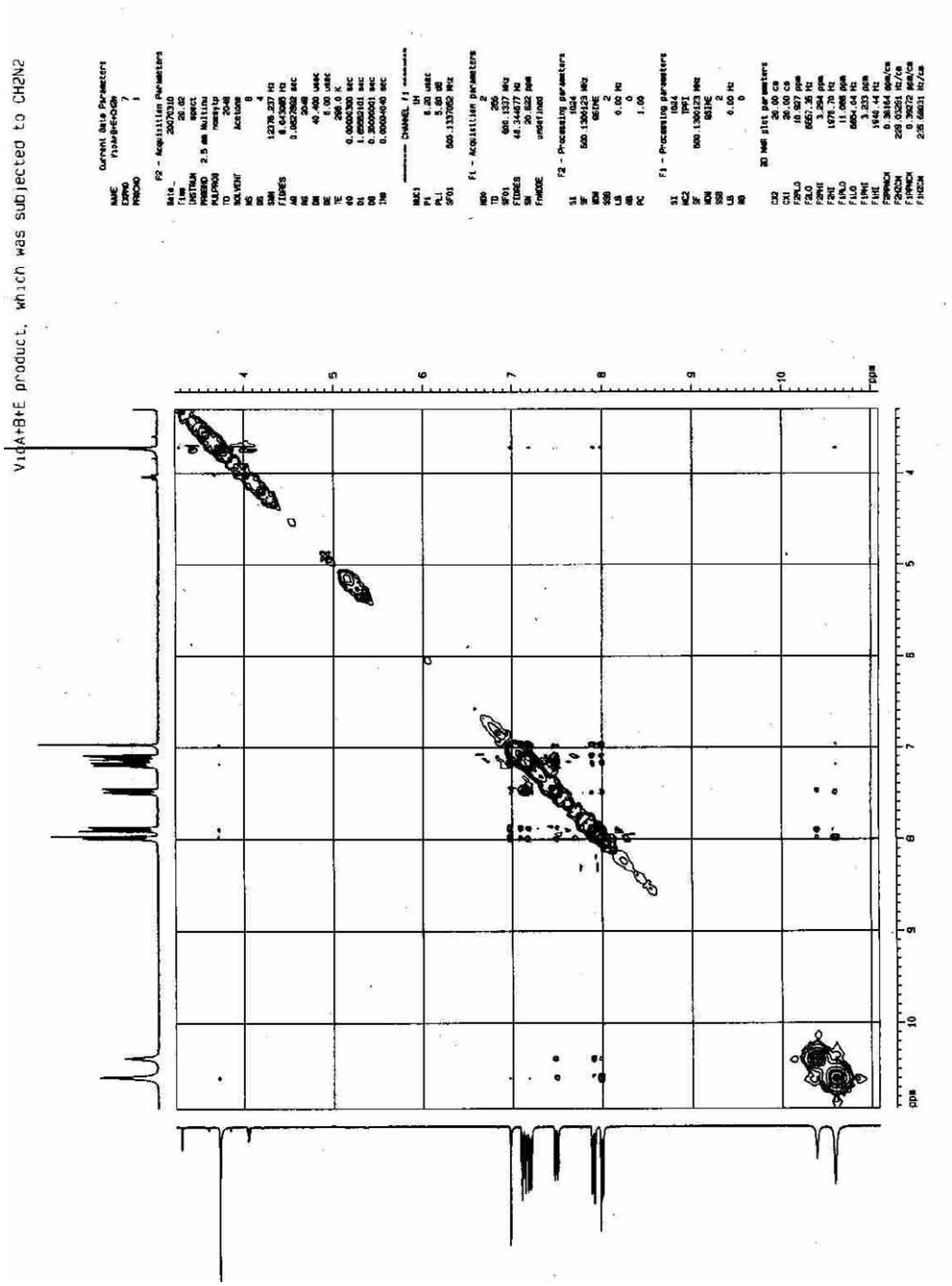
COSY 90



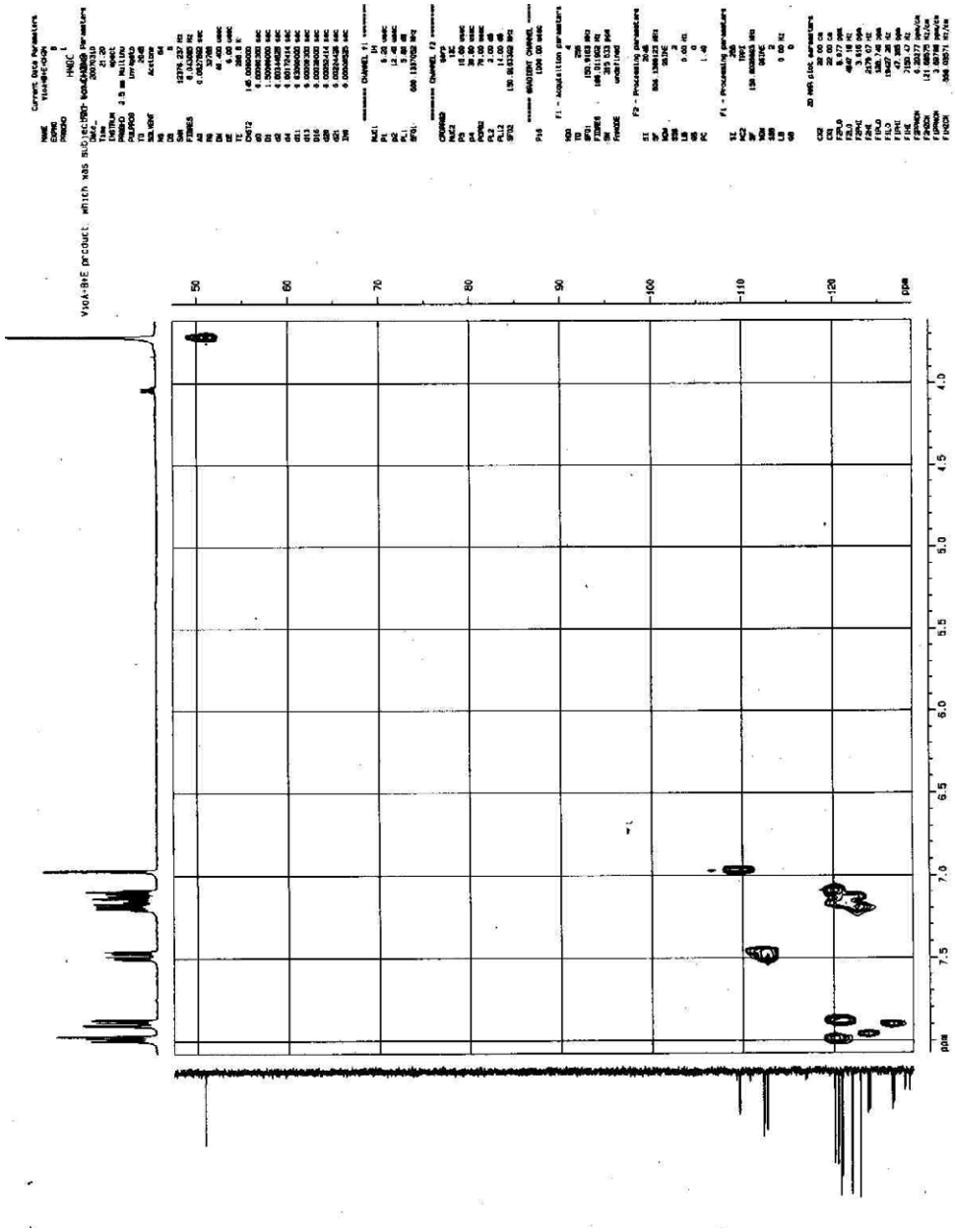
HOHAHA



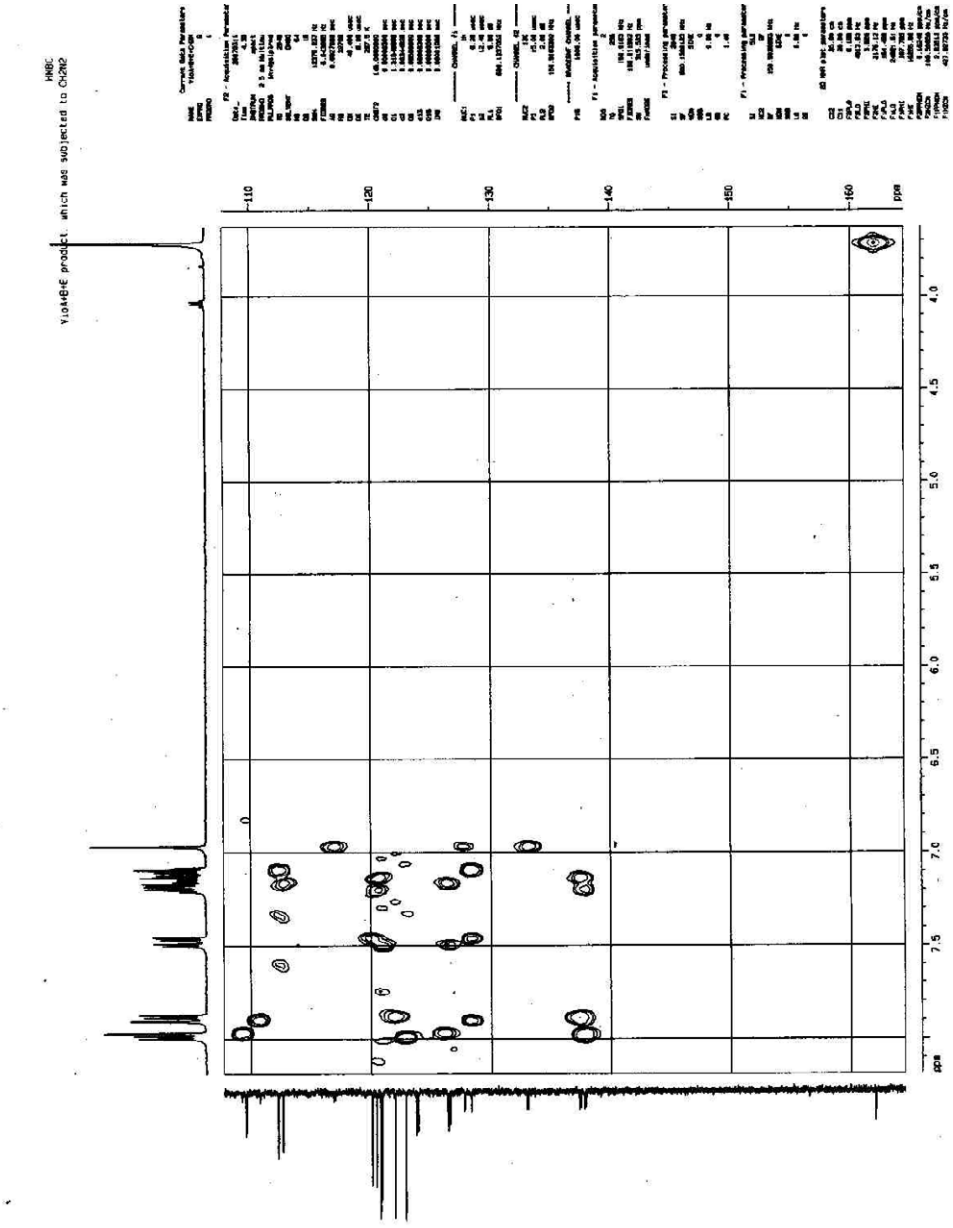
NOESY



HMQC

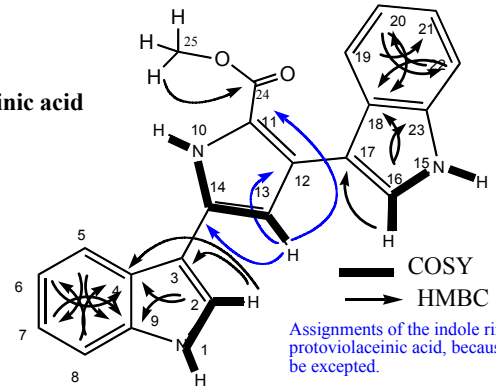


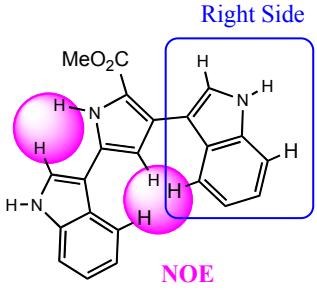
HMBC


HMBC
Yio4B4E product which was svojected to CH2N2
110
120
130
140
150
160
ppm
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
ppm

17. NMR assignment of methyl ester of 9

Me ester of protodeoxyviolaceinic acid





Assignments of the indole ring at the right side were based on those of the protoviolaceinic acid, because the essentaily same chemical shifts should be excepted.

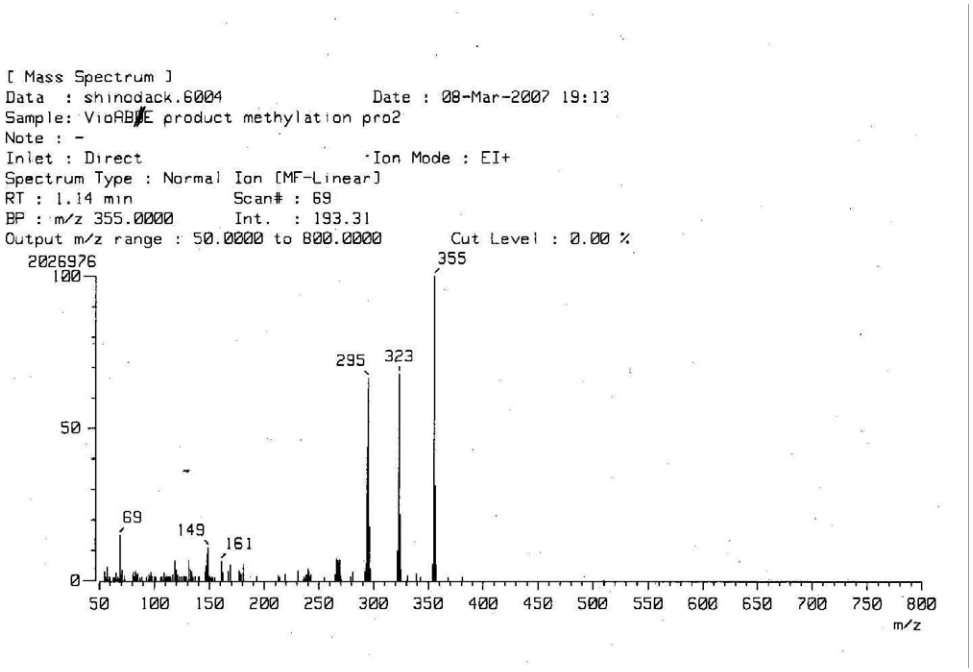
Measured at 23°C (room temp.) in acetone-d_6 with 400 MHz δ_H and δ_C were given in relative to the solvent peaks of 2.04 ppm and 29.8 ppm

No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C	No	^{1}H	^{13}C
1	10.57(brs)	—	6	7.18(m)	120.9	11	—	127.7[a]	16	7.91(d, 2.4Hz)	126.6	21	7.13(t, 7.9Hz)	121.9
2	7.97(d, 2.8Hz)	123.9	7	7.20(m)	122.8	12	—	116.9[a]	17	—	110.7	22	7.47 (d, 7.9Hz)	112.2
3	—	109.2	8	7.51(d, 7.6Hz)	112.6	13	6.98(d, 2.8Hz)	109.5	18	—	128.2	23	—	137.4
4	—	126.3	9	—	137.8	14	—	133.0	19	7.89(d, 8.0Hz)	120.7	24	—	162.0
5	7.99(d, 7.7Hz)	120.4	10	10.60(brs)	—	15	10.37(brs)	—	20	7.09)t, 7.9Hz)	120.1	25	3.72(3H,s)	50.78

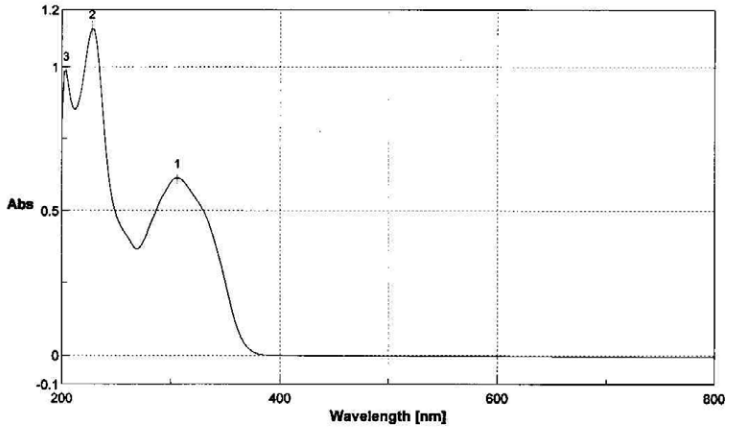
a: The assignments may be exchangeable.

18. EI-LRMS and EI-HRMS of methyl ester of 9.

HREIMS, $C_{22}H_{17}N_3O_2$, Calc. 355.1321, Found, 355.1319.



19. Electronic spectrum of methyl ester of 9



Optical pathlength; 1 cm, Concentration 0.638 mg/50 ml MeOH

20. Homologous alignment of VioA , VioC and VioD

VioA;

```
vioA                                                                    418 a.a.
L-amino acid oxidase [Synechococcus elongatus PCC 6301].                495 a.a.
           identities 15.5% positives 32.1% gaps 18.1%
L-amino acid oxidase [Trimeresurus stejnegeri].                         516 a.a.
           identities 14.4% positives 33.1% gaps 20.0%

vioA              ----------------------------------------------MKHSSDICIVGAG
Synechococcus     --MRFSRRRFLQSSLGAAATTG--LAGTLAAGGQAQTRSTPVR-------KRSVLVLGAG
Trimeresurus      MNVFFMFSLLFLAALGSCADDRNPLEECFRETDYEEFLEIARNGLKATSNPKHVVIVGAG
                                                                    : ::***

vioA              ISGLTCASHLLDSPACRGLSLRIFDMQQEAGGRIRSKMLDGKASIELGAGRYSPQLHPHF
Synechococcus     MAGLTAALSLLR----RGHQVTVIEYQNRIGGRLLSVPLKG-GQFSEAGGGHFRANMPYV
Trimeresurus      MSGLSAAYVLAG----AGHEVTVLEASERAGGRVRTYRNDEEGWYANLGPMRLPEKHRIV
                  ::**:.*  *       * .: ::: .:. ***: :   .  .     .          .

vioA              QSAMQHYSQKSEVYP------FTQLKFKSHVQQKLKRAMN----ELSPRLKEHGKESFLQ
```

```
Synechococcus       LSYIRHFKLPLLTLN--DGLPRYLFDGKTADAADLSRWP----WDLAPQERRVSVASLLN
Trimeresurus        REYIRKFNLQLNEFSQENDNAWHFVKNIRKTVGEVKKDPGVLKYPVKPSEEGKSAEQLYE
                     . ::::.              ..      .:.:       : * .  .  .: :

vioA                FVSRYQGHDSAVGMIRSMG--YDALFLPDISAEMAYDIVGKHPEIQSVTDNDANQWFAA-
Synechococcus       TYLILNGLDTDTVLDANWPDAQAIQQLDNLTLSQLIRQVGGSEAFIQLLDAHGGTFTSSS
Trimeresurus        ESLRKVEKELKRTNCSYILNKYDTYSTKEYLIKEGNLSPGAVDMIGDLMNEDAGYYVSFI
                           :                 :   .     *   : .: : ... : :

vioA                --------------ETGFAGLIQG---IKAKVKAAG--ARFSLGYRLLSVRTDGDGYLLQ
Synechococcus       PALGVIPDLAYHFGDQNLFRIQGGNDRLPKAMAAAIGSERFILDAPVVAIDQQANRATVT
Trimeresurus        ESMKHDDIFAY---EKRFDEIVDGMDKLPTSMYRAI-EEKVHFNAQVIKIQKNAEEVTVT
                                : : : *  :  : *   :. :.  :: :  :.:   :

vioA                LAGDDGWKLEHRTRHLILAIPPSAMAGLNVDFPEAWSGARYGSLPLFKG----FLTYGEP
Synechococcus       VK----DGRTFQGDAIISTIPFTVLPEVAVRPGWSAGKRRMFAEMEWEQTVKVIAQTRSP
Trimeresurus        YHTPEKDTSFVTADYVIVCTTSRAARRIKFEPPLPLKKAHALRSVHYRSGTKIFLTCTKK
                              :*  . .  : .   .   :     :.    :   .

vioA                WWLDYKLDDQ-VLIVDNPLRKIYFK------GDKYLFFYTDSEMANYWRG-CVAEGEDGY
Synechococcus       VWLAQNVHGWPMAGSDRPWERVIDITGNEGGGYGNTFFYLNGRNKDAMLARPKSERAQAI
Trimeresurus        FREDEGIHGG-KSTTDLPSRFIYYPNHNFTSGVGVIIAYGIGDDANFFQALDLKDCGDIV
                          :..     * * . :       *   : * .  :  .   : :

vioA                LEQIRTHLASALGIVRERIPQPLAH----VHKYWAHGVEFCRDSDIDHPSALSHRDSGII
Synechococcus       VDQFRSDLPDLFDEVVTLADFAWGEQPWIRGSFGG------PPLGGAWMIREWTTPEGLI
Trimeresurus        INDLS-LIHQLPREEIQTFCYPSMIQKWSLDKYAMGGITTFTPYQFQHFSEALTSHVDRI
                    :::: : .          .        .:                      . *

vioA                ACSDAYTEHCG-WMEGGLLSAREASRLLLQRIAA-------------
Synechococcus       HFAGDFTTMKSGWVEGAIESGLRAARQIDPGAQPEADTFLRQEQRCN
Trimeresurus        YFAGEYTAHAHGWIDSSIKSGLTAARDVNRASENPSGIHLSNDDEL-
                      :. :*    *::..: *.  *:* :
```

VioC

Kmo : kynurenine 3-monooxygenase from *Homo sapience* (identity; 26%, positives 47%, gap; 4%)

Phbh : *p*-hydroxybenzoate hydroxylase from Pseudomonas ptida (identity 18%, positive 34%, gap 7%)

Large letters: anino acid residues

```
                        GXGXXG
VioC:  ----------MKRAIIVGGGLAGGLTAIYLAKR-GYEVHVVEKRGDPLRDLSSYVDVVSSRAIGVSMTVRGIKS
Kmo :  ---MDSSVIQRKKVAVIGGGLVGSLQACFLAKR-NFQIDVYEAREDTR-----VATFTRGRSINLALSHRGRQA
Phbh:  ---------MKTQVAIIGAGPSGLLLGQLLHKAGIDNVILERQTPDYVLG--------RIRAGVLEQGMVDLLR
                     VhhhGsGhhGhhhs
VioC: VLAAGIPRAELDACGEPIVAMAFSVGGQYRMRELKPLEDFRPLSLNRAAFQKLLNKYANLAGVRYYFEHKCLDVD
Kmo : LKAVGLEDQIVSQGIPMRARMIHSLSGKKSAIPYGTKSQYILSVSRENLNKDLLTAAEKYPNVKMHFNHRLLKCN
Phbh: EAGVDRRMARDGLVHEGVEIAFAGQRRRIDLKRLSGGKTVTVYGQTEVTRDLMEAREACGATTVYQAAEVRLHDL
                                DG
VioC: LDGKSVLIQGKDGQPQRLQGDMIIGADGAHSAVRQAMQSG------LRRFEFQQTFFRHGYKTLVLPDAQALGYR
Kmo : PEEGMITVLGSDKVPKDVTCDLIVGCDGAYSTVRSHLMK-------KPRFDYSQQYIPHGYMELTIPPKNGDYAM
Phbh: QGERPYVTFERDGERLRLDCDYIAGCDGFHGISRQSIPAER-----LKVFERVYPFGWLGLLADTPPVSHELIYA
                              chhhssDGxcSxhR
VioC: KDTLYFFGMDSGGLFAGRAATIPDG---SVSIAVCLPYSGSPSLTTTDEPTMRAFFDRYFGGLP--RDARDEMLR
Kmo : EPNYLHIWPRN--TFMMIALPNMNK---SFTCTLFMPFEEFEKLLTSND-VVDFFQKYFPDAIP--LIGEKLLVQ
Phbh: NHPRGFALCSQ------------------RSATRSRYYVQVPLTEKVEDWSDERFWTELKARLP--AEVAEKLVT
                                GD
VioC: QFLAKPSNDLINVRSSTFHYKGNVLLLGDAAHATAPFLGQGMNMALEDARTFVELLDRHQGDQD---KAFPEFTE
Kmo : DFFLLPAQPMISVKCSSFHFKSHCVLLGDAAHAIVPFFGQGMNAGFEDCLVFDELMDKFSNDLS---LCLPVFSR
Phbh: GPSLEKSIAPLRSFVVEPMQHGRLFLAGDAAHIVPPTGAKGLNLAASDVSTLYRLLLKAYREGR-----GELLER
                              GxhhLhGDAAHxxxPxxGxGxNxsxxDsxxL
VioC: LRKVQADAMQDMARANYDVLSCSN--PIFFMRARYTRYMHSKFPGLYPPDMAEKLYFTSEPYDRLQQIQRKQNVW
Kmo : LRIPDDHAISDLSMYNYIEMRAHVNSSWFIFQKNMERFLHAIMPSTFIPLYTMVTFSRIRYHEAVQRWHWQKKVI
Phbh: YSAICLRRIWKAERFSWWMTSVLHR-----FPDTDAFSQRIQQTELEYYLGSEAGLATIAENYVGLPYEEIE---


VioC: YKIGRVN-------------------------------------------------------
Kmo : NKGLFFLGSLIAISSTYLLIHYMSPRSFLCLRRPWNWIAHFRNTTCFPAKAVDSLEQISNLISR
Phbh:-----------------------------------------------------------------
```

Small letters : h=hydorphobic, c=charged, s=small, x=all, -=gap

VioD.

salicyl-CoA 5-hydroxylase [Streptomyces sp. WA46] 517a.a.

identities 23.9% positives 39.3% gaps 29.2%

2-polyprenyl-6-methoxyphenol hydroxylase and related FAD-dependent oxidoreductase [Marinobacter sp. ELB17].

411 a.a.

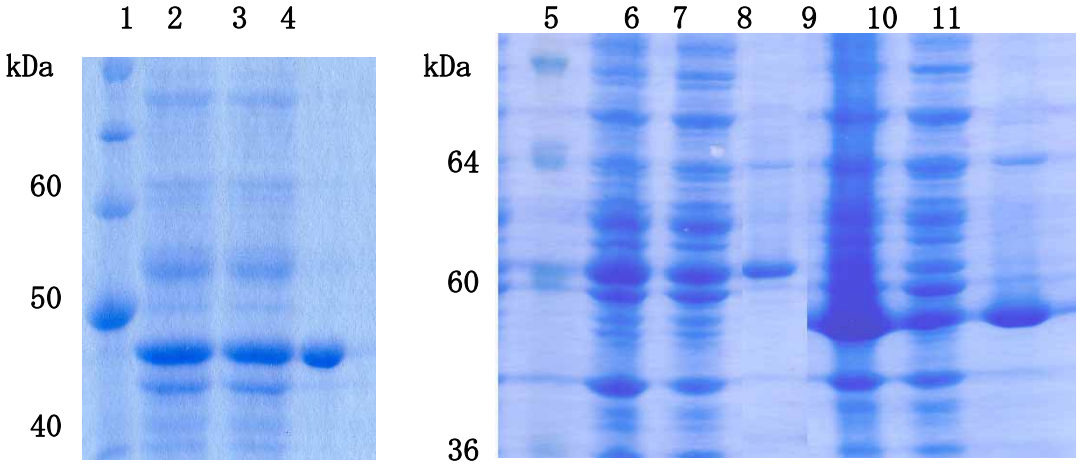
identities 16.2% positives 35.5% gaps 12.3%

```
vioD        ----MKILVIGAGPAGLVFASQLKQ-------------------ARPLWAIDIVEKNDEQ
salicyl     ----MKVACIGAGPGGLFFATLLKR-------------------SRPDAEVVVFERNRPD
2-poly      MTQAFDIAVVGAGMVGAALATGLGRNGFRVALIDFADAPEFVPGSTPDIRVSALSAGSER
                :.:  :***  *  :*: * :                  : *   :  .. .

vioD        EVLGWGVVLPGRPG-QHPANPLSYLDAPERLNPQFLEDFKLVHHNEPSLMSTGVLLCGVE
salicyl     DTFGFGVVFSDATL-DAIDAADPVLSEALEKHGRHWDDIEVRVHGERERVG-GMGMAAVV
2-poly      YLQGLKAWDNVLAMRATPYRRLAVWDQSSHPLTRLLPKPLARVEFNADKLG-ASHLGHIV
              * .    .        . . . .  :  .    . : . :. .  :  :

vioD        RRGLVHALRDKCRSQGIAIRFESPLLEHGELPLADYDLVVLANGVNHKT-AHFTEALVPQ
salicyl     RKTLLSLLQERARAEGVQMRFQDEVRDP--AELDDFDLVVVCDGANSRFRTLFADDFGPT
2-poly      ENSVTQAALWQAASTQPNITLMPGVAVTNLTQNHDHAQLDLSNATAITATLVVGADGAQS
            .. :     :. :   : :  :        *.  : :.:..       .

vioD        VDYGRNKYIWYGTSQLFDQMNLVFRT-HGKDIFIAHAYKYSDTMSTFIVECSEETYARAR
salicyl     AEVASAKFIWFGTTYMFDGLTFVHQD-GPHGVFAAHAYPISDSLSTFIVETDADSWARAG
2-poly      RVRDLAGIGVTRSQYDQQAMVMSVRYRGAVEDITWQGFLPSGPRAFLPLHTAGEEFPGES
                       :   : : :  :      : :.:  *.. : : :.   : :.

vioD        LG--------EMSEEASAEYVAKVFQAELGGHG-----LVSQPGLGWRNFMTLSHDRCHD
salicyl     LDAFDPATPLGMSDEKTKSYLEDLFRAQIDGHP-----LVGNNSR-WANFATRRARSWRS
2-poly      WGSLVWYDAPARLAQLKAMPIEQLMAEIQQEFPQPLPELTHIDTRASFPIARQHAKHYYQ
             .           : .   : .::     .     *.        :        .

vioD        GKLVLLGDALQSGHFSIGHGTTMAVVVAQLLVKALCTEDG------VPAALKRFEERALP
salicyl     GKWVLLGDAAHTAHFSVGSGTKMAMEDAVALAETLGEASRS-----VPEALDLYEERRRP
```

```
2-poly          GRVVLAGDAAHTINPLAGQGVNLGFQDAQCLQQLLIVARNNGTDLADPDLLSQYEHQRRP

vioD            LVQLFRGHADNSRVWFETVEERMHLSSAEFVQSFDARRKSLPP----------------
salicyl         KVERIQNSARPSLSWWEHFGRYVRSFDAPTQFAFHFLTRSIPRGKLAVRDAAYVDRVDGW
2-poly          ANRRMMLAMDVFYHLFSNRVPPLHLLRNLGLGAAQALPFARNR----------------

vioD            -----MPEALAQNLRYALQR---------------------------------------
salicyl         WLRHHEAGPLKTPFRVGPYRLPTRRVTVGDDLLTGTDGTGIPMVPFSGQPFGAGVWIDAP
2-poly          ----------VARYAMGLD---------------------------------------

vioD            ------------------------------------------------------------
salicyl         DTEEGLPLALDQVRETAEAGVLLVGVRGGTALTRVLVAEEARLAHSLPAAIVGAYDDDTA
2-poly          ------------------------------------------------------------

vioD            ----------------
salicyl         TTLVLSGRADLVGGTK
2-poly          ----------------
```

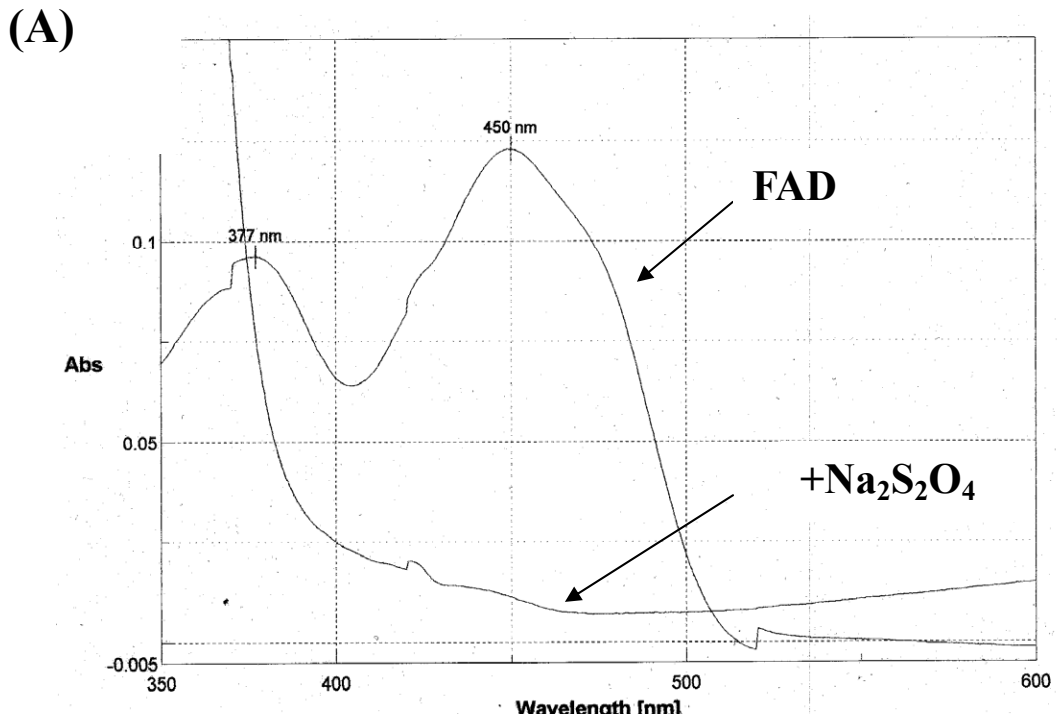
21. FAD cofactor involved in VioA, C and D.

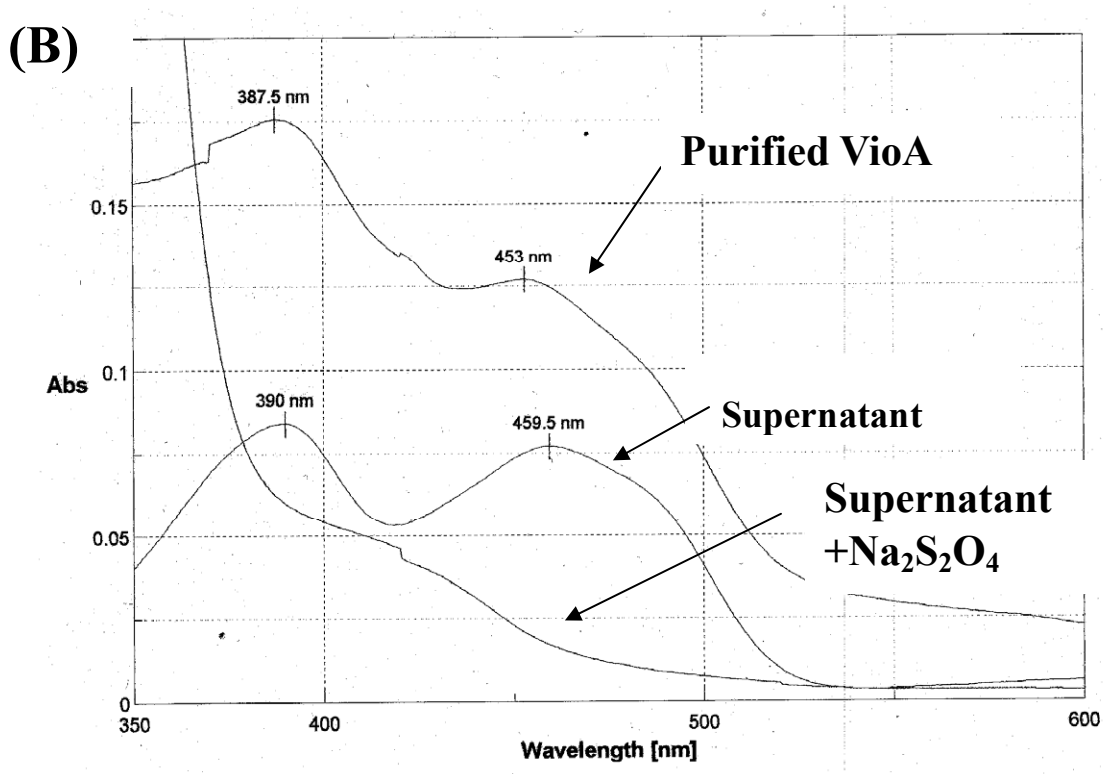
21-1: SDS-PAGE of the expressed proteins and the purified VioACD



Lane 1 :MW marker
Lane 2 : pET16b-vioA, total protein
Lane 3 : pET16b-vioA, soluble protein
Lane 4 : purified VioA with Ni-NTA
Lane 5 : MW marker
Lane 6 : pET16b-vioC, total protein
Lane 7 : pET16b-vioC,soluble protein
Lane 8 : Purified VioC with Ni-NTA
Lane 9 : pET16b-vioD, total protein
Lane 10 : pET16b-vioD soluble protein
Lane 11 : purified VioD with Ni-NTA

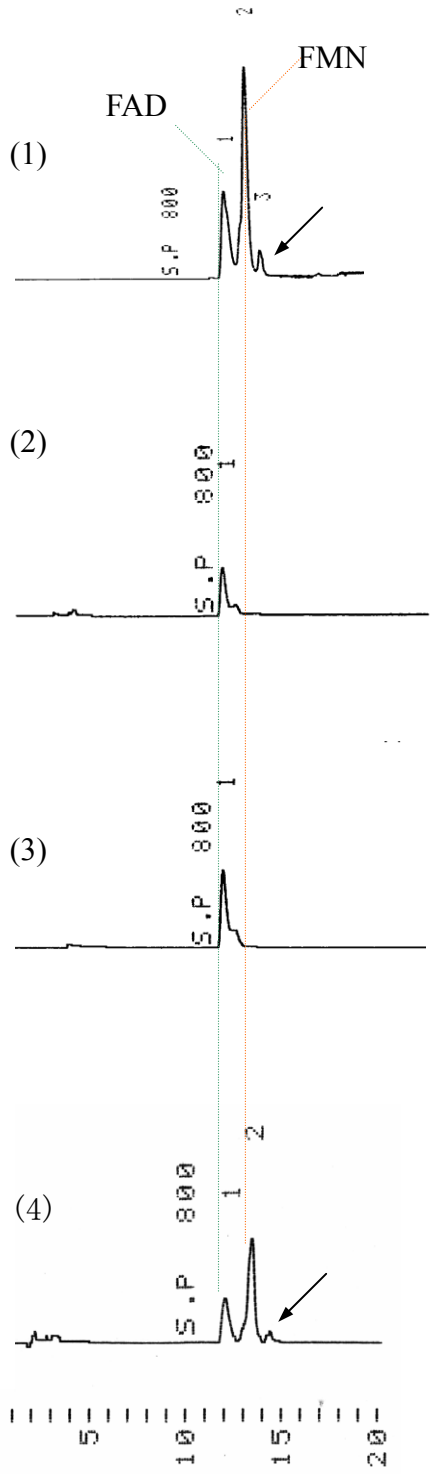
21-2: Electronic spectra of authentic FAD (A) and the supernatant (B) obtained by heating the purified VioA.





The similar electronic spectra were also obtained for VioC and VioD.

21-3: HPLC profiles of the supernatant obtained after heating the purified VioC at 100°C for 10 min.



HPLC conditions**.** Column; SHISEIDO, CAPSELL PACK C18 (Type:MG 5 μm), 4.6 mm I.D. x 150 mm; detected at 448 nm. The HPLC patterns were obtained according to the following gradient program.

Table Gradient Program

	min	Solvent A (%)	Solvent B(%)	Flow(ml/min)
(1)	0	100	0	0.8
(2)	0.5	100	0	0.8
(3)	17.5	75	25	0.8
(4)	18.0	100	0	0.8
(5)	23	100	0	0.8

Solvent A：15 % MeOH/6mM H_3PO_4、Solvent B：MeOH

(1): sample injection

(2) and (3): 1.25 % MeOH linear gradient

(4) and (5): the column washing, then back to (1)

Arrow indicates the impurity involved in the commercially available FMN.

(1) Co-injection of authentic FAD and FMN.

(2) The supernatant obtained by heating the purified VioC

(3) Co-injection of the supernatant and authentic FAD

(4) Co-injection of the supernatant and authentic FMN.

The similar HPLC analyses showed that VioA and VioD were also a flavo-protein containing FAD, but not FMN.

The ratio of each of VioA and VioD to FAD was determined to be nearly 1.0. To the cell-free extracts of VioA and VioD, which were obtained from 100 ml-culture, was added 0.5 mg FAD/10 ml of the cell-free extract, and incubated for 15 min at 25°C. After purification with Ni-NTA column, the purified VioA and D were heated at 100°C for 10 min. The FAD content of the supernatant was determined by the HPLC described above. However, in the case of VioC, the released FAD amount of the supernatant was very low even after the preincubation with the supplemented FAD, the ratio of the released FAD to VioC was ca 0.2:1. This result suggests that FAD may more tightly bind to VioC. Further study is necessary to clarify this point.