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Isolation and Characterisation of Compound from *Stachytarpheta cayennensis* (Rich.) Vahl Leaves

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Abstract

The structure of compound isolated from *Stachytarpheta cayennensis* leaves was elucidated using a combination of IR, ^{1}H and ^{13}C NMR, COSY, DEPT NMR, Mass spectral and comparison with similar structure found in literature. The chloroform extract of the plant leaves yielded a compound proposed as Nnenoside B. The compound was isolated at the ratio of 80:20 (pet.ether:chloroform) of elution mixtures of column chromatography and the purity monitored with TLC, which yielded a single spot with pet.ether/chloroform/methanol ratio of 55/30/15 (Rf = 0.893) was observed. As a follow-up on the study, therefore, it is anticipated that the compound can further be explored to reveal its potential applications.

Keywords

Active Compound, Characterisation, Column Chromotography, Isolation, Plant, TLC

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1. Introduction

The use of plants or their extracts all over the world predates the introduction of modern pesticides, drugs and other industrial applications and had remained an integral part of both pest control and primary health care (Nick *et al.,* 1995; Anesini and Perez, 1993; Desta, 1993 and Liu, 1987) as well as source of other industrial materials. Traditional medical practitioners in Nigeria, use a variety of herbal preparations to treat different kinds of diseases.

Plants contain untapped reservoir of bioactive compounds that can be used directly as well as 'lead' compounds for synthetic compounds. These compounds have wide applications such as found in natural pesticides of plant origin which their active agents have been developed and are currently in use. These include Calabar bean (*Physostigma venenisum)*, used traditionally as an ordeal poison from where methyl carbamate insecticides were developed; and pyrethrum insecticides from the flower of *Chrysanthemum cinerariaefolium* extract, which was discovered because of its local use to control insect pest (Oldfield, 1984). The root of *Lonchocarpus* is a source of rotenone used as poison to stun fish (Plotkin, 1988).

This therefore points out that the whole parts of the plant: fruits, flowers, leaves, stems, bark and roots can be potential source of active agents which can be employed for diverse uses. These parts of plant contain secondary metabolites known as phytochemicals of which extracts can contain active compounds that have potentials for use in the development of natural active products (Agte *et al*, 1999; Dev and Karl, 1997; Saxena and Kidiavai, 1997 and Okogun 1983).

Stachytarpheta cayennensis (Verbenaceae Family) is an herb commonly found in Nigeria as a weed of waste places, anthropogenic sites, roads and weeds of field crops with long growing season. It is not recognized and regarded as an important plant. The plant's common names are: the blue rats tail or rough-leaved false vervian (English); *Iru amure* (Yoruba), *Wulsigai Kusu* (Hausa), *Oke nchụ anwụnta ọhịa* (Igbo) (Akobundu and Agyakwa, 1998), *Opa para* (Abeokuta).

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Stachytarpheta cayennensis is an erect, shrubby perennial plant up to 1.5m high that produces seeds. The stem is four angled, woody at the base and has slender branching stems that are covered with short hairs. The leaves are opposite or sometimes alternate with short, winged stalks. The blades are ovate to elliptic in shape, about 8 cm long and 4 cm wide hairy on both surface and prominently net veined with rounded or pointed apex, evenly toothed margins and contracted at the base (Akobundu and Agyakwa, 1998). The inflorescence is a long slender, hair spike, about 20 cm long, occurring at the shoot terminal. The flowers are white or lilac with white centres, sessile with 4-5 mm long and five lobed petals (2 large and 3 small) about 4-5mm in diameter. The seed is a two-seeded kernel or nutlet enclosed by a persistent calyx that is embedded in a shallow groove in the inflorescence axis.

Stachytarpheta cayennensis is known to possess pesticidal activity, in its local applications as a mosquito repellant. It is also used to repel insects in farms to avoid damages to crops such as melon. The plant has been used by different localities as a remedy to many ailments. It is used widely in Nigeria for the treatment of dysentery and as a remedy for gonorrhea, ulcer etc. The juice is used to cure eye troubles and sores in children's ears. The macerated leaves and roots have been claimed to treat sore skin wounds. There are also some side effects such as vomiting, loss of appetite and unconsciousness etc (Akobundu and Agyakwa, 1998)

It is the ultimate aim of this research to isolate and characterise the bioactive compound in *Stachytarpheta cayennensis* locally used to control pest so as to ascertain their potentials as sources of natural pesticides which may further serve as lead compounds for the synthesis of synthetic prototype based on the characterisation of the isolated compounds.

2. Materials and Methods

Starchytarpheta cayennensis plant was harvested from an uncultivated area of Michael Okpara University of Agriculture Umudike, Ikwuano Local Government Area of Abia State, Nigeria. The plant was identified by Mr. Ibe Ndukwe of the Forestry Department, Michael Okpara University of Agriculture, Umudike, Abia State.

2.1. Preparation and Preservation of Samples

The plants samples were harvested and the leaves were separated from their stem and some with flowers were left with the stem. The leaves were air-dried at room temperature. The air-dried samples were pulverized into powdery form using brand new corona landers manual milling machine which were then stored in labelled polyethene bags.

2.2. Extraction of Sample

The extraction method used in this study was a modification of JohnBull (2001a and b) and JohnBull and Abdu (2006). 750 g of the pulverized samples were percolated in about 2.5 litres of ethanol (98% analytical standard) for 72 hours to obtain the ethanol (crude) extract, followed by filteration and then concentrated under reduced pressure rotary evaporator at 34^{o}C (JohnBull *et al*, 2001a and b). These were then allowed to stand for complete evaporation of the remaining ethanol at room temperature. The crude ethanol extracts were stored in labelled covered beakers for further analyses.

2.3. Partitioning of the Crude Ethanol Extract

Part of the crude ethanol extracts of the samples was subjected to partitioning. 20 g of the crude ethanol extract of the sample was partitioned using 150ml/150ml v/v of chloroform and water. The sample was first dissolved using the solvents. This was continuously stirred to ensure complete dissolution and was then transferred into a separating funnel which was shaken until homogenous mixture was obtained. It was then allowed to stand for 24 hrs before separating. The chloroform extracts was allowed to evaporate completely under room temperature. The crude ethanol extract was fractionated as below. The samples were subjected to preliminary Thin Layer chromatography (TLC) was used to determine the number of compounds that could be present in each of the extracts.

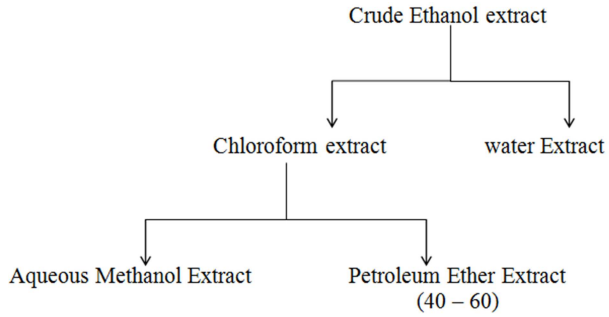

Fig. 1. Schematic diagram of the partitioning process.

2.4. Column Chromatography of Chloroform Fraction

Three (3.0) g of chloroform fractions of *Starchytarpheta cayennensis* leaves was mixed with 30 g of silica gel (50 – 200 mesh) to get homogenous solid mixture slurry and loaded on a silica gel packed column. 3 g of silica gel was then added to protect the sample applied. The column was eluted with different solvent mixture gotten from the manipulation of the ratio mixture, starting with 100% (100

ml) petroleum ether. Then varying the mixture ratio at 5 ml interval with chloroform ie (95 ml pet.ether and 5 ml chloroform, followed by 90 ml pet.ether and 10 ml chloroform) etc, until it got through to 100% (100ml) chloroform. Thereafter, methanol was mixed in like manner with chloroform until there was complete elution. The eluates were collected in fraction of 100 ml. Each fraction was evaporated to dryness and transferred into a labelled 100 ml beaker and covered with foil. The fractions were monitored on TLC and the one that gave single spots was selected and set aside for spectroscopic analyses.

IR was recorded on Perkin Elmer FT Model at Covenant University, CanaanLand Ota. Readings were taken between 4000 cm^{-1} and 625 cm^{-1}. Mass Spectrometry (MS) was performed on a Finnigan MAT Incos-XL mass spectrometer (San Francisco, CA) operating under electron impact (EI) ionization mode at 70 eV, scanning from *m/z* 33 to 750 in 0.59 s.

^{1}H NMR spectra were recorded on a Bruker AM-500 FT-NMR spectrometer operating at 500 MHz, while ^{13}C NMR (broad band and DEPT; proton decoupled at 125 MHz on a Bruker AM-500 spectrometer, Germany and UK). The ^{13}C NMR spectral assignment was made partly through DEPT and partly through a comparison of the chemical shifts with the published data for similar compounds. The purity of the compounds was monitored on TLC with silica gel.

Distortionless Enhancement of Polarization Transfer (DEPT): The DEPT technique has proven superior to others in providing information on attached protons reliably, efficiently and with high selectivity. It is a proton-carbon polarization transfer method, so DEPT spectra are actually more sensitive than normal acquisitions. A set of spectra with pulse delays adjusted for B/2 (DEPT-90) and 3B/4 (DEPT-135) were taken. The DEPT-90 spectrum shows only CH carbons, the DEPT-135 shows positive CH_3 and CH, and negative CH_2 signals. It is important to understand that the appearance of positive and negative signals can be reversed by phasing, so it is necessary to have some way of determining whether the spectrum has been phased for CH_2 positive or negative. Quaternary carbons are invisible. "Leakage" can occur in DEPT-90 spectra because 1*J*C-H varies as a function of environment, and the technique assumes that all 1*J*C-H are identical. This can result in small peaks for CH_2 and CH_3 signals, which should have zero intensity. For similar reasons the C-H of terminal acetylenes (C/C-H) will show anomalous intensities in DEPT spectra (either nulled or very small in DEPT-90, or present in DEPT-135) because the CH coupling is much larger (around 250 Hz) than the normal value of 125 Hz for which the DEPT experiment is usually parameterized. To further prove this structure and to assure that these assignments were correct, data were compared with ^{13}C-NMR data in literature for compounds with similar structures.

3. Results and Discussion

3.1. Partitioning, TLC and Column Chromatography

The results of the partitioning and preliminary TLC analyses are shown in Table 1. The ethanol extracts of the leave of the plants percolated yielded more five fractions on partitioning. It was clear that the fraction contained more one spots on the TLC.

Table 1. Results of Chromatography of partitioned extracts and Crude extracts and number of spots observed

Sample code	Solvent fraction	Weight of sample (g)	Nature/number of spots in TLC	Solvent ratio used in TLC
SCL0.1	Ethanol	37.84	2	90/10 C/P
SCL0.2	Chloroform	5.26	3	90/10 C/P
SCL0.3	Water	-		
SCL0.4	Pet.ether	3.21	2	90/10 C/P
SCL0.5	Aqueous methanol	-		

KEY: Stachytarpheta cayennensis leaves (SCL) C = chloroform, P = pet. ether

3.2. Column and Thin Layer Chromatography of Chloroform Fraction of SCL0.2

The result of the 3.0 g of the chloroform fraction of the *Stachytarpheta cayennensis* leaves that was chromatographed in a small column of silica gel using different solvents for the elution, which include pet.ether, chloroform and methanol, and mixed at small intervals to get different polarities for the elution gave signs of elution immediately with 100% pet.ether and because of this, the polarities of the eluent used were varied at a very close range. The order of variation in the polarities of the solvent and TLC for the fractions obtained are shown in Table 2. From the table, thirty five fractions were obtained and six fractions showed clearly single spots. Fractions eluted at 90/10 and 85/15 chloroform/methanol ratios were alike and were suspected to be isomers. However, the fraction eluted at 80/20 of chloroform/methanol was finally selected for spectral analysis because it gave a single spot on the TLC which is a sign of purity of the compound isolated with that fraction.

The result of the partitioning and TLC analysis of this fraction is shown in Table 2.

Table 2. Result of Column and Thin Layer Chromatography of Chloroform Fraction of SCL0.2

Column eluents Mixture rates		TLC Solvent ratio	No. Of Spots/shape	Rf values	Column eluents Mixture rates		TLC Solvent Ratio	No. of spots /shape	Rf values
Pet. ether	Chloro-form				Chloro-form	Meth-anol			
100	0	-	-	-	95	5	70/22.5/7.5 C/P/M	1	Too small
95	5	-	-	-	90	10	As above	1*	
90	10	-	-	-	85	15	As above	1*	
85	15	-	-	-	80	20	55/30/15 C/P/M	1	0.893
80	20	97.5/2.5 C/P	1	0.780	75	25	As above	Multiple	
75	25	92.5/7.5 C/P	MWS	-	70	30	As above	Dragged	
70	30	As above	2	-	65	35	As above	Dragged	
65	35	As above	2	-	60	40	As above	Dragged	
60	40	As above	1	0.909	55	45	As above	Dragged	
55	45	As above	1	0.909	50	50	70/25/5 C/P/M	Dragged	
50	50	As above	Flame		45	55	As above	Dragged	
45	55	90/10 C/P	1	0.515	40	60	As above	Dragged	
40	60	70/22.5/7.5 C/P/M	MWS	-	35	65	As above	Dragged	
35	65	As above	2	-	30	70	As above	Dragged	
30	70	As above	2	-	25	75	As above	Dragged	
25	75	As above	MWS	-	20	80	As above		
20	80	As above	NWD	-	15	85	70/30 C//M	-	
15	85	As above	NWD	-	10	90	As above	-	
10	90	As above	MWS	-	5	95	As above	-	
5	95	As above	2	-	0	100	-	-	
0	100	As above	1	0.709					

Key: C = chloroform, P = pet. ether, M = methanol, * suspected to isomers but not well clear; MWS = Moved with solvent front; NWD = Not well differentiated

The partition of crude ethanol extract of *Stachyterpheta cayennensis* leaves using equal volumes of chloroform and water provided a lipophilic and hydrophilic fraction. The lipophilic was separated using column chromatography on silica gel. The eluates were monitored using TLC of varying polarities. This fraction was separated at ratio of 80:20 (pet.ether:chloroform) elution mixture of the column chromatography and was observed as a single spot on the TLC with pet.ether/chloroform/methanol ratio of 55/30/15 (Rf = 0.893).

The Infrared Spectrum for the vibrational frequency of the compound (proposed as Nnenoside B) isolated from the leaves of *Stachyterpheta cayennensis* showed absorption peaks characteristic bands at V_{max} 2980cm^{-1} and 2750cm^{-1} being prominent peaks evident for C-H stretching in unsaturated aromatic compounds, unsaturated compounds (alkenes) and aliphatic groups. The carbonyl frequency of absorption was observed at 1700cm^{-1}. Undoubtedly, carbonyl functional groups are easily observed between 1670 – 1780cm^{-1}. The other characteristic absorption peaks observed also include V_{max} 1620cm^{-1} which is mostly for C=C, N- H of unsaturated aromatic and heterocyclic groups. Aromatic ring system usually show characteristic peaks between 1600 and 1500cm^{-1} (Dudley and Ian, 1980). The finger print portion of the IR of the compound showed a large number of absorptions peaks due to variety of single bond vibration. The absorption peak of 1380cm^{-1} could be attributed to the C-O – C stretching motion of ether and sugar. Also, other functional groups observed are listed in Table 3.

Table 3. Infrared Absorption Bands for the Compound from *Stachyterpheta cayennensis* leaves

Absorptions Peaks (cm^{-1})	Group	Remarks
3345	N-H	Secondary Amines
3005	= C-H , C = C	Aromatic str vibration
2980	-CH_2 – CH_2-, CH_3	Aliphatic C-H stretching
2750		Aliphatic groups
1700	C=O	Carbonyls
1620	C = N, N-H, C = C	Unsaturated aliphatic, aromatics, unsaturated heterocyclic, amines
1450	CH_3 and CH_2	C-H bending or deformation
1380 -1240	C – O – C. C -O – H	Ethers, alcohols, sugar
720		Substituted Aromatics compounds

Table 4. ^{1}H NMR Chemical Shift of the Compound from the leave of *Stachyterpheta cayennensis*.

∂-Shift	No of protons	Multiplicity	Assignment
0.8	3H		
1.3	2H		H
1.5	3H	s	H-2/4/12/15/18/19/20
1.6	1H	t	H-1
2.0	2H	t	H-3
2.3	2H	t	H-22
2.8	2H	s	H-28/29 and sugar
3.0 – 4.0	Poorly resolved	s	H-18
4.3	3H	s	Sugar
5.5	1H		O-CH_3
7.3	1H	s	N-H, O –H, H-7
7.6	1H		Ar-H,
7.8	1H		

Table 5. ^{13}C NMR Chemical Shift of the Compound from the leave of *Stachyterpheta cayennensis*.

Carbon No./Assignment	∂-Shift	Multiplicity	Carbon No./Assignment	∂-Shift	Multiplicity
1	22	CH_2	24		C
2	32	CH_2	25		C
3	77	CH	26	130.7	CH
4	43	CH_2	27	128.7	C
5	35	CH	28	128.9	OCH_3
6	18	CH_2	29	133	OCH_3
7	124.5	CH	$1^{/}$	59.4	CH
8	124.7	C	$2^{/}$	55.5	CH
9	55	C	$3^{/}$	77	CH
10	36	C	$4^{/}$	77	CHO
11	77	CH	$5^{/}$		CH
12	22	CH_2	$6^{/}$	59	OCH_3
13	39	C	$7^{/}$		OCH_3
14	77	C	$8^{/}$		OCH_3
15	29	CH_2	$1^{//}$		CHO
16	125	C	$2^{//}$		CH
17	125.2	C	$3^{//}$	53	CH
18	78	CH_2	$4^{//}$		CH
19	59	CH_2	$5^{//}$	59	CH
20	59	CH_2	$6^{//}$		OCH_3
21	19	CH_3	$7^{//}$		OCH_3
22	39	CH_2	$8^{//}$		OCH_3
23	183	C	$9^{/}$		OCH_3

The ^{1}H NMR (Table 4) showed chemical shifts at ∂ 0.8 ppm which are protons of the methine group in cyclic system. It also displayed signals due to influence of ring system in the compound (Rubinstein *et al.*, 1976). The methyl group at C-21 showed prominently as a singlet at ∂ 1.5. The other chemical shifts are characteristics of steroidal skeleton and sugar moiety.

The ^{13}C NMR (Table 5) spectral assignment was made partly through Normal and DEPT. The ^{13}C NMR of Nnenoside B exhibited chemicals shift for aromatic group at ∂ 124.5, 124.7, 125, 125.2, 133, 130.7, 128.7, 128.9 and 129.2 for C-7, C-8, C-16, C-17, C-23, C-23, C-24, C-25, C-26 and C-27 respectively. In addition, inspection of ^{13}C NMR revealed the presence of signal at ∂ 77, 59, 53 ppm corresponding to sugar moiety. The DEPT was conclusively used to assign carbon shift. A combination of ^{13}C and DEPT 135 NMR showed the compound contains one methyl, nine methylene, fifteen methine and nine methoxy, four methinoxy and ten quartenary carbons.

This compound also displayed diagnostic signals at the finger print region of IR which is a characteristic of CH and CH_2 protons in the steroid nucleus.

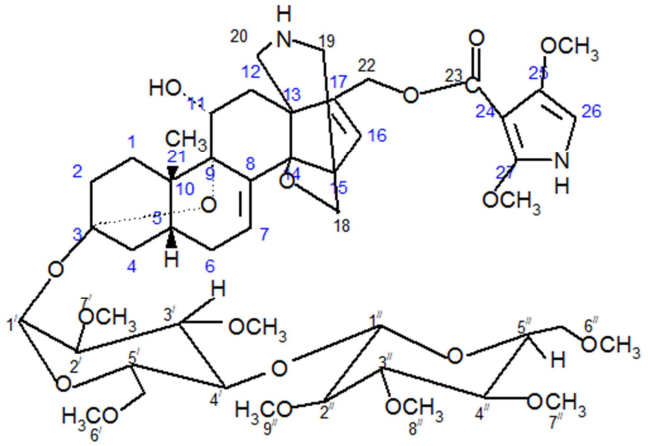

Fig. 2. Nnenoside B

Then ^{13}C- ^{13}C COSY was not deprotonated in effect, the resonance of the proton splitting pattern as well as carbon splitting pattern are highly complicated correlation pattern which made the spectra highly difficult to interpret. Much interpreted spectra are proton decoupled whereby the carbon and proton are separated to give different spectra.

This was further substantiated by the mass spectral analysis gave a molecular ion peak of m/z 887 which suggested a molecular formula of $C_{54}H_{56}O_{18}N_2$ with a base ion peak at m/z 554.1788. The peak observed at m/z 931 could be attributed to the presence of impurity. A loss of m/z 377 of the sugar moiety resulted in a fragment ion peak at m/z 505. However, further cleavage and subsequent loss of a sugar moiety of m/z 225 gave rise to the fragment peak ion at m/z 668. The other possible cleavages and fragments are illustrated in Fig. 3. All these information were used to propose the structure below.

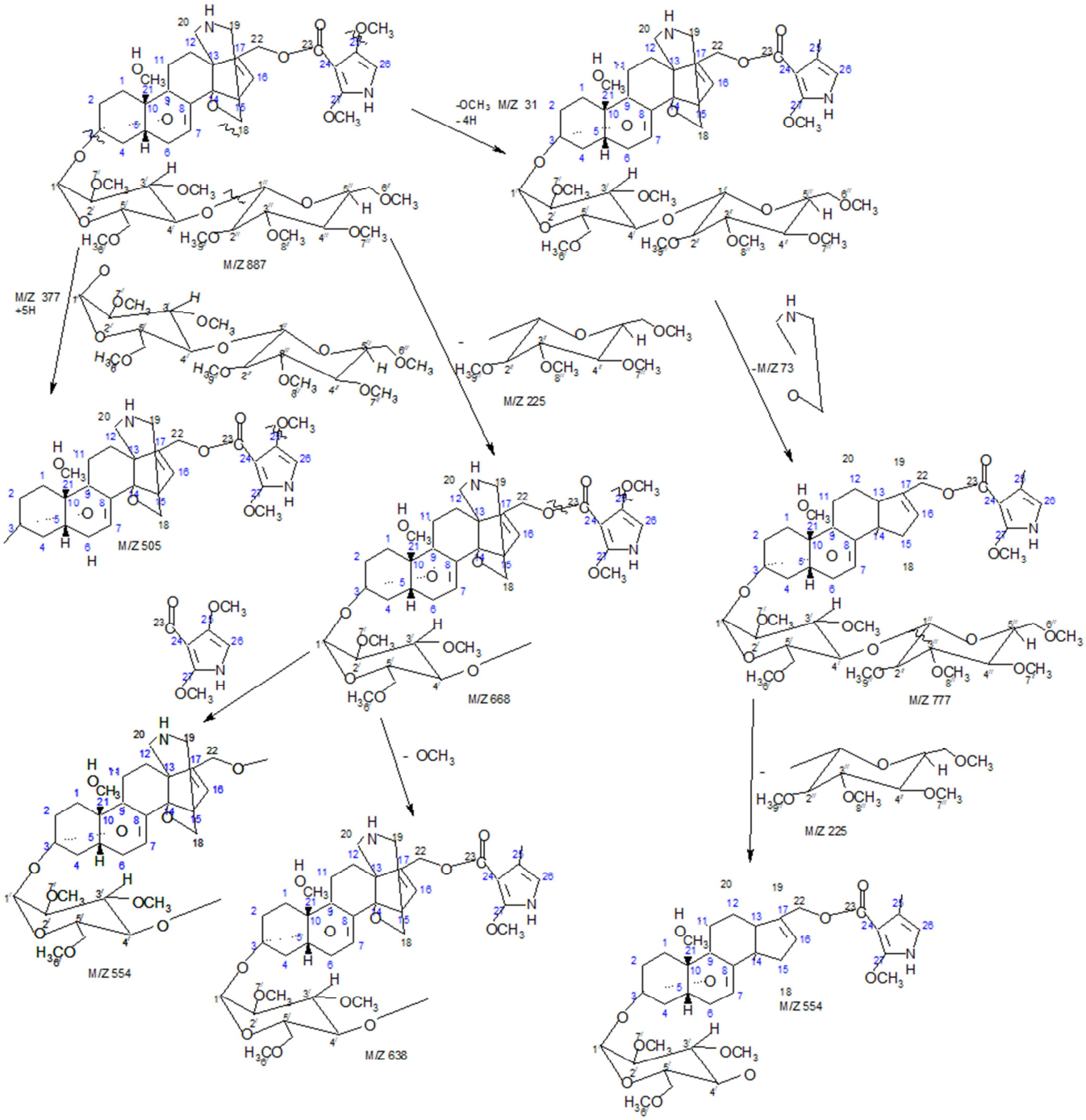

Fig. 3. Fragmentation Pattern of Nnenoside B.

Similar compound with steroidal nucleus has been isolated from the leaves and stem bark of *Stachyterpheta jamiacensis* linn vahl (Okwu and Ohenhen, 2010; Okwu and Ohenhen, 2009a and b) and from the root of *Tetrapluera Tetraptera* (Okoronkwo *et al.*, 2012)

4. Conclusion

The isolated compound from *Stachytarpheta cayennensis* known to possess mainly pesticidal activity among its other local applications, was proposed as Nnenoside B, with its structure containing a steroidal nucleus. However, many steroidal based compounds of plant origin have also been isolated from the different parts of plants which include the leaves, stem bark and root. Therefore, the compound can further be explored for its potentials applications in different areas.

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