UNIVERSITY OF CAPE COAST

EXTRACTION OF ALKALOIDS FROM THE STEM OF THE PLANT

CRYPTOLEPIS SANGUINOLENTA (LINDL.) SCHLTR.

BY

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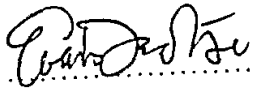
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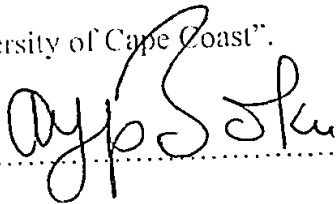
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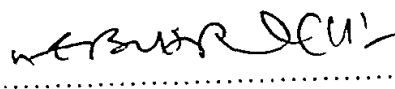
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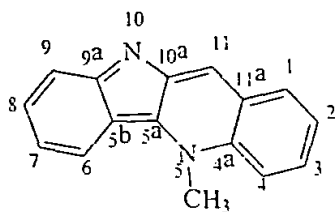(Dr. Y. Opoku-Boahen)

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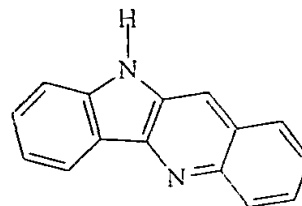**Co-Supervisor.**

(Mr. V.Y.A Barku)

ABSTRACT

The roots of *Cryptolepis sanguinolenta* have been investigated for their chemical compounds since 1931. But so far, not much has been reported on the stem although they are used in traditional medicine in Ghana. *Cryptolepis sanguinolenta* (Lindl.) Schlechter (family Periplocaceae) is a medicinal plant used in some African countries against infectious and parasitic diseases. Phytochemical screening of the extracts from the stem (bark and wood) of *Cryptolepis sanguinolenta* revealed the presence of mainly alkaloids, which were detected and confirmed by the Mayer's, Dragendorff's and Wagner reagents.

Chromatographic separation methods (Column, Preparative TLC, and TLC) of the methanol (Alkaloid extract) yielded two isolates, whose structures were elucidated using spectroscopic methods. The purity of these alkaloids was ascertained by means of thin layer chromatography. The final structures of these compounds were then deduced by comparison of the experimental data with those proposed in literature. They are both indoloquinolines. The first one [1.11] (CH2) was identified as cryptolepine. The second isolate [1.12], (CH4), has been named as quindoline (indolo[3,2-b]quinoline). The two compounds are among the most common of the indoloquinoline alkaloids extracted from the plant.

[1.11]

[1.12]

ACKNOWLEDGEMENTS

The writing of this thesis could not have been possible without the help of many people, Firstly, I would like to extend my sincere thanks to my supervisor, Dr. Yaw Opoku-Boahen for his excellent supervision, endless patience, optimism and encouragement towards the development of this work. Messers: V. Y. A. Barku and J.P.K. Adotey deserve my sincere thanks for the assistance and words of encouragement they gave me.

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Finally, to a very special friend Faustina Halm-Lai for being there for me.

God bless you.

DEDICATION

This work is dedicated to my uncle.

Kobla Harry Nyaledzigbor.

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CHAPTER ONE

INTRODUCTION

The plant, *Cryptolepis sanguinolenta* (Lindl.) Schltr. (Asclepiadaceae) belongs to the family Periploaceae, although it had earlier been classified as a member of the family Asclepiadaceae. The new family to which the plant belongs can be distinguished from the Asclepiadaceae by the presence of granular pollen borne on spathulate granular carriers (Hutchinson and Dalziel, 1963). *Cryptolepis* belongs to the order Apocyales. The genus, *Cryptolepis* includes *Cryptolepis brazzei*, *C. deciduas*, *C. oblongifolia*, *C. nigritana*, *C. triangularis* and *C. sanguinolenta*, which is common in West Africa. It is also known as *Pergularia sanguinolenta* Lindl. (Hutchinson and Dalziel, 1963).

The plant is given various names in different parts of the country. "Gangamau" (among the Hausa speaking people), "kadze" (among the Ewe), and "nibima"(among the Twi speaking people). The plant is also known as the "Ghana Quinine" or yellow-dye root. Although the aqueous extract has a bitter taste, this name is probably based on the common use of the plant as a substitute for the anti-malarial alkaloid quinine, and should not be confused with it (HerbalGram, 2003).

The plant *Cryptolepis sanguinolenta* grows wild along the West coast of Africa (Ghana, Nigeria, Guinea-Bissau and Senegal) and some parts of Central

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Africa (Congo-Brazzaville). Although it grows wild, it can also be cultivated. It avoids the wet rain forest where there is abundant shade. It thrives well in areas where there is adequate supply of sunshine and water; thus it is completely absent from the mushy and salty swamps of coastal regions. In Ghana, the plant is commonly found in the month of June in certain districts of the country. On the Aburi hills, in the Akwapim area, it is found in areas where there is enough rainfall for optimum growth. Around Ejura district of the Ashanti region, the plant thrives well in the wooded savannah vegetation, reaching a peak of abundance in June as it pertains in the Aburi hills (Hutchison and Dalziel, 1963).

It is a thin-stemmed twining and scrambling shrub. The leaves are petiolate, glabrous, elliptic or oblong-elliptic, up to 7 cm wide. The blades have an acute apex and a symmetrical base. The inflorescence cymes, lateral on branch shoots, are few flowered, with a yellow corolla tube up to 5 mm long. The fruits are paired in linear follicles and are horn-like. The seeds are oblong in shape, small (averaging, 7.4 mm in length and 1.8 mm in the middle), and pinkish, embedded in long silky hairs. The dried leaves, stems and roots of *Cryptolepis* have a sweet fragrance. The root varies from 0.4-6.6 cm long and 0.31-1.4 cm wide and has a bitter taste. The roots are rather tortuous and branch with little or no rootlets. The outer surface is yellowish brown and when dry, show longitudinal ridges with occasional cracks. The roots break easily with fractures leaving a smooth transverse surface, which is yellow in colour. The sap is extremely bitter and is characterized by the rapidity with which it turns deep red on exposure to air (Sofowora, 1984; Boye, 1992).

Fig.1: Photograph of the plant *Cryptolepis sanguinolenta*, (Courtesy of the Centre for Scientific Research into Plant Medicine, Mampong-Akwapim, Ghana).

Ethnobotanical uses of the Plant

Cryptolepis sanguinolenta is known to have a lot of uses including medicinal ones. The plant is known to be used by Ghanaian traditional healers in the treatment of various fevers; including malaria (Boye and Ampofo, 1983). The roots of *Cryptolepis* have been formulated and used as tea bags in the treatment of acute uncomplicated malaria. The herbal tea (Phyto-laria®) is formulated by the Phytomedicine Division, of Phyto-Riker Pharmaceuticals, (Accra, Ghana), (Boye, 2002).

The safety and efficacy of (Phyto-laria®) as a treatment for malaria has been demonstrated in a clinical trial conducted by the above mentioned Pharmaceutical Company. Phyto-laria®, the cryptolepis product formulated as a tea, has been evaluated *in vivo* by administering it orally to mice, rats, and rabbits and using the conventional acute toxicity and clinical chemistry tests. This tea bag formulation has been shown to be safe (Cimanga *et al.*, 1996; Luo *et al.*, 1998). The LD_{50} (lethal dose in which 50 percent of test animals died) obtained was above 2,000mg/kg, more than two orders of magnitude higher than the effective dose (HerbalGram, 2003). Since then, the herbal tea (Phyto-laria®) has been approved by the Food and Drugs Board, and is packaged with instruction on the volume of boiling water to use per tea bag (Boye, 2002; NMIMR, 2002).

In Ghana, the dried root decoction is used in traditional medicine to treat various forms of diseases including fever, malaria, urinary and upper respiratory tract infection, rheumatism, and venereal diseases (Booakye-Yiadom, 1979; Boye and Oku-Ampofo, 1983; Wright *et al.*, 1996). The roots of this plant have been

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used in the form of a decoction in the clinical therapy of malaria, urinary tract infections, and upper respiratory tract infections at the Centre for Scientific Research into Plant Medicine at Mampong-Akwapim, Ghana since 1974 (Boye and Oku-Ampofo, 1983).

Cryptolepis is also used in Congolese traditional medicine for the treatment of amoebiasis. An aqueous decoction of the root bark of *Cryptolepis* is used in the Congo for this treatment (Tona *et al.*, 1998). Research focused on malaria led to the identification of alkaloids, principally cryptolepine the major alkaloid of the plant, as its antimalarial agent (Karou *et al.*, 2003; Banzouzi *et al.*, 2004).

A systematic phytochemical investigation of the plant has led to the isolation of several indolo[3,2-b]quinoline derivatives, related to the parent compound quindoline (Dwuma-Badu *et al.*, 1978; Tackie *et al.*, 1993; Dhanabal *et al.*, 2006; Sharaf *et al.*, 1995; Cimanga *et al.*, 1996).

In the Democratic Republic of Congo and the Casamance District of Senegal, infusions of the roots are used in the treatment of stomach and intestinal disorders and rheumatism (Kerharo and Adem, 1974; Sofowora, 1984; Boye and Oku-Ampofo, 1990). The root is sold and used as a yellow dyestuff amongst the Hausa of Northern Nigeria and other parts of West Africa and South Angola (Sofowora, 1984). It has been used as a dye in the leather industry in Ghana for many years (Dalziel, 1956; Saxton, 1965; Oliver-Bever, 1986).

The plant has shown to be important in Guinea-Bissau where the Fulani traditional healers use it to treat jaundice and hepatitis (Boye and Ampofo, 1983).

Also, the root decoctions of this plant has been used by traditional healers for the treatment of various fevers and hepatitis, and the leaves have been used for treatment of malaria or powdered as a cicatrizant of wound (Feijão, 1961; Gomes and Diniz, 1993). The folks of Guinea-Bissau use the aqueous extracts of the plant *Cryptolepis sanguinolenta* in the treatment of urinary tract infections caused by *Candida albicans, Neisseria gonorrhoea* of *Escherichia coli* (Boakye-Yiadom, 1979). In the Guinea-Bissau, the crude extract of *Cryptolepis sanguinolenta* is known to inhibit the growth of strains of *N. gonorrhoea, C. albicans* and *E. coli* (Boakye-Yiadom, 1979).

Out of 12 plants used in Guinea-Bissau traditional remedies to treat infectious diseases, only cryptolepis was found effective against *Escherichia coli* and nine out of the 10 microbial test organisms used; only *Pseudomonas aeruginosa* was not susceptible (Silva *et al.*, 1996). The ineffectiveness of *Cryptolepis sanguinolenta* against *P. aeruginosa* has also been reported in another anti-microbial screening study *in vitro*, using extracts as well as five of the alkaloids isolated from *Cryptolepis sanguinolenta* (Paulo *et al.*, 1994).

Research Objectives and Significance

The plant *Cryptolepis sanguinolenta* appears to be well known among the local people, of the West African sub-region, who often use it in the treatment of fever, malaria, urinary and upper respiratory tract infections, and venereal diseases. In view of its widespread use as a local medicine, the plant would appear to merit study. It was decided as a step in this direction to determine the

chemical constituents, which could be isolated in a pure state. Moreover, the stem

was selected because, not much work has been done on it. Whilst there is more

information on alkaloids from the other parts of the plant especially, the roots,

there is little or no information on the alkaloids in the stem.

CHAPTER TWO

LITERATURE REVIEW

General Review of Indoloquinoline Alkaloids

Distribution of Quindoline Alkaloids

Indolo[3,2-b]quinoline [1.12] is sometimes referred to in literature as Quindoline. It belongs to a group of compounds called indoloquinoline alkaloids. The indoloquinoline alkaloids are not widely distributed in nature. They have been identified only in a few families of plants. Quindoline [1.12] was first isolated from Cryptolepis species (*C. triangularis* and *C. sanguinolenta*) all of the Periplocaceae family (Dwuma-Badu *et al.*, 1978). The compound has also been isolated from other plants such as *Sida acuta* (Malvaceae) from Sri Lanka (Gunatilaka *et al.*, 1980), *Microphilis guyanensis* (Sapotaceae), *Genipa americana* (Rubiaceae) from Surinam (Yang *et al.*, 1999) and also from the leaves of *Justicia betonica* (Gottumukkala *et al.*, 2004).

Nomenclature of Indoloquinoline Alkaloids

Indoloquinoline alkaloids consist mainly of the indole [2.10] and the quinoline [2.11] moieties fused through carbon. Both the indole and the quinoline nuclei are contained in several groups of alkaloids with important physiological properties such as antimalarial activity.

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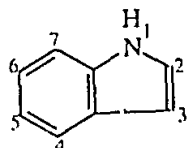
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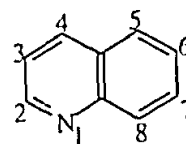
[2.11]

Quinoline [2.11] is a heterocyclic compound in which a benzene ring [A] and a pyridine ring [B] are fused through carbon (Gilchrist, 1992). Indoles on the other hand, are also heterocyclic compound in which a benzene ring [A] and a pyrrole ring [B] are fused through a carbon (Norman, 1968). The two moieties then join to each other via the five-membered nitrogen-containing pyrrole (B) and six-membered nitrogen-containing compound pyridine (B1) respectively to form the alkaloid, for example quindoline [1.12]. In other words they are fused at the second and third carbon positions respectively in forming indolo[3,2-b]quinoline.
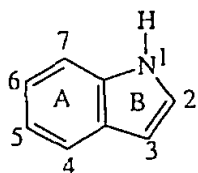
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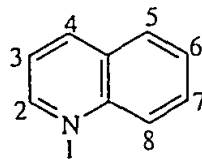
[2.11]

Chemical Constituents of *Cryptolepis sanguinolenta*

Alkaloids have mainly been isolated from the plant *Cryptolepis sanguinolenta*. As at 1996, it was reported that about 15 alkaloids had been extracted from the plant *Cryptolepis sanguinolenta* and out of these, seven of the isolated alkaloids are novel, not having been isolated previously from nature nor having been described as a synthetic product, while the remaining 8 alkaloids are

of an incompletely or partially determined structure as at the present time (Sharaf et al., 1996).

The indoloquinoline alkaloid Cryptolepine (5-methyl-5H-indolo[3,2-b]quinoline [1.11] was first isolated from extracts of the roots of *Cryptolepis triangularis* N.E.Br., a species native to the Belgian Congo, and synonymous with *Cryptolepis sanguinolenta* (Delvaux, 1931). Cryptolepine [1.11] isolated from Nigerian sample of *Cryptolepis sanguinolenta* (Gellert et al., 1951). Cryptolepine [1.11] with a molecular formula of $C_{16}H_{12}N_2$ has a melting point of 166-169°C (Gellert et al., 1951).

Almost 30 years later, the alkaloid was isolated from Ghanaian sample of *Cryptolepis sanguinolenta* (Dwuma-Badu et al., 1978; Dwuma-Badu, 1987; Ablordeppey et al., 1990; Cimanga et al., 1991; Tackie et al., 1991) along with quindoline [1.12] (norcryptolepine) and a partially characterized alkaloid (CSA-3). A new alkaloid, 5H-indolo[3,2-c]quinoline named isocryptolepine [1.17] which is reported by Pousset et al (1994) is believed to be the partially characterized alkaloid CSA-3 (Dwuma-Badu et al., 1978).
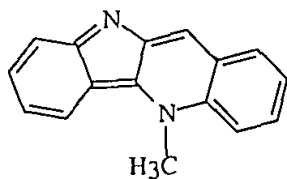
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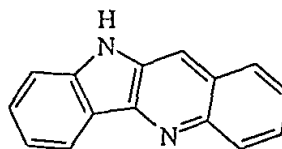
[1.12]

Recently, the isolation of a third alkaloid from this species, a unique spiro nonacyclic alkaloid, Cryptospirolepine, [1.13] has been reported (Tackie et al., 1993; Pousset et al., 1995). Cryptospirolepine an indoloquinoline alkaloid maybe biogenetically derived from cryptolepine [1.11] (Tackie et al., 1991).

Three new indole alkaloids, hydroxycryptolepine, [1.14] cryptoheptine, [1.15] and cryptoquindoline, [1.16] were also reported (Paulo et al., 1994; Paulo et al., 1995).

[1.13]

[1.14]

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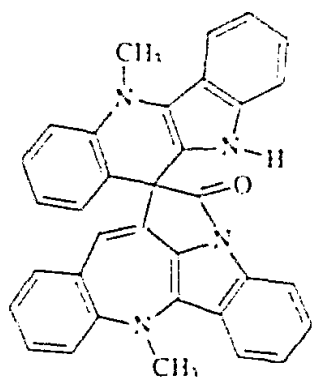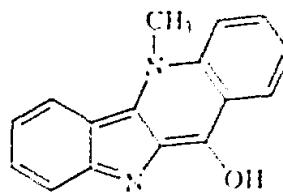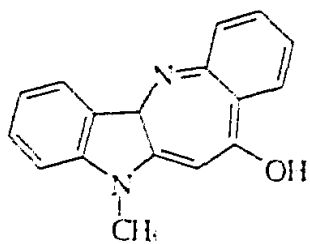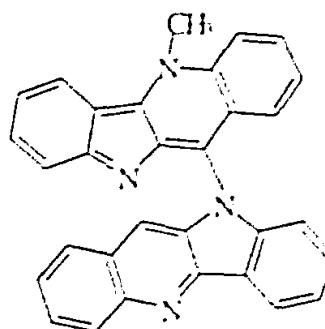
In 1995 the isolation of a new alkaloid isocryptolepine [1.17] an isomer of cryptolepine [1.11] was reported (Crouch *et al.*, 1995; Pousset *et al.*, 1995; Cimanga *et al.*, 1996). Not quite long ago. Cimanga *et al.* (1996) reported on the characterization of two new alkaloids from the root bark of the same plant *Cryptolepis sanguinolenta*. These alkaloids were biscryptolepine [1.18] and neocryptolepine [1.19] (also referred to as crytotackieine). Cryptomisrine [1.20] an example of a dimeric indolo[3,2-b]quinoline alkaloid was the first to have been isolated from nature, have been reported (Sharaf *et al.*, 1996). Crytomisrine is most unusual in that its two monomeric parts apparently exist in such a C_2 symmetric environment that only one set of proton and carbon nmr resonances are observed (Sharaf *et al.*, 1996).

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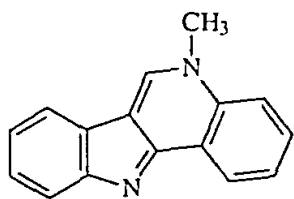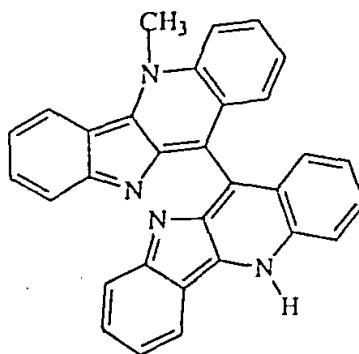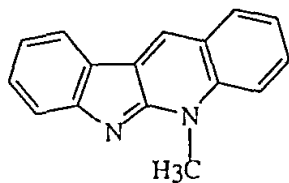
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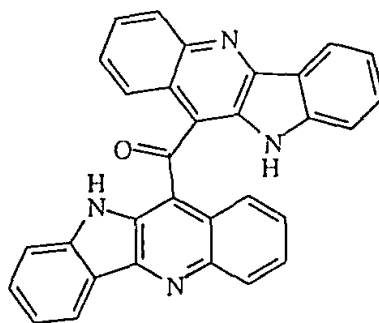
[1.20]

Very recently, cryptolepinone and the mixed dimer cryptoquindolinone [1.21] were identified as two of the 26 chromatographically separable degradation products of cryptospirolepine [1.13] that formed on long term storage of a NMR sample in DMSO-d6 (Martin *et al.*, 2002). Cryptolepinone and its oxidation product, the 5-N-oxide, were isolated and identified previously in a series of reports (Sharaf, 1993; Cooper *et al.*, 1998; Sharaf *et al.*, 1998; Hadden *et al.*, 1999; Martin *et al.*, 1999). The extraction and structure elucidation of the quindoline derivative compound quindolinone (5H, 10H-indolo[3,2-b]quinolin-11-one) [1.22] have also been reported (Crouch *et al.*, 1995).

[1.21]

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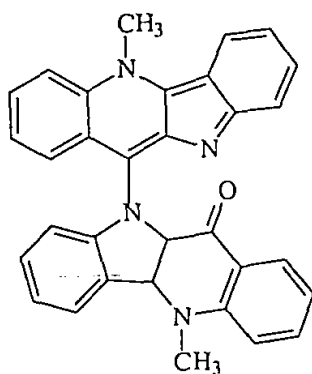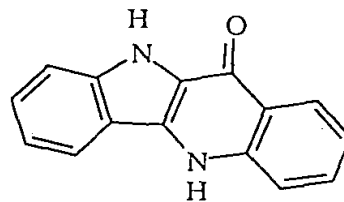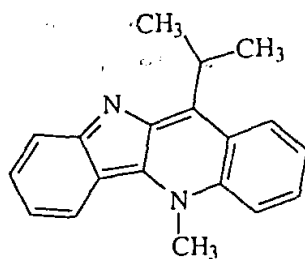
[1.23]

In 1999, another new alkaloid isolated from the roots of the plant *Cryptolepis sanguinolenta* was reported. This new alkaloid 11-isopropylcryptolepine [1.23] has an unusual incorporation of an isopropyl group at the 11-position of the indolo[3,2-b]quinoline nucleus. This is suggestive of a mixed biosynthetic origin for the alkaloid (Hadden *et al.*, 1999).

Pharmacological Activities of Indoloquinoline Alkaloids

Cryptolepine [1.11] an indoloquinoline, high yields of which are extracted from the roots of the West African shrub, *Cryptolepis sanguinolenta*, has been tested for various biological activities.

Of all the isolated alkaloids, cryptolepine [1.11] is the most active anti-bacterial agent, and it is more active against Gram-positive bacteria than the Gram-negative bacteria (Boakye-Yiadom, 1979; Cimanga *et al.*, 1991; Sawer *et al.*, 1993; *Paulo el al.*, 1994a).

Cryptolepine [1.11] has some anti-fungal activity against *Saccharomyces cerevisiae* but not the *Candida* species (Sawer *et al.*, 1995). Its anti-fungal activity seems to be limited compared to its anti-bacterial activities. Cryptolepine [1.11] has also been tested for *in vitro* antiplasmodial activity against the multi-drug

resistant (K1) strain of *Plasmodium falciparum* and found to be highly active (Noamesi *et al.*, 1991; Kirby *et al.*, 1995; Wright *et al.*, 1996; Grellier *et al.*, 1996; Cimanga *et al.*, 1997).

In another study, with multi-drug resistant K1 strain of *P. falciparum* and a method of assessing inhibition of parasite growth based on measurement of lactate dehydrogenase activity, it was shown among a number of anhydronium bases, only cryptolepine [1.11], the major alkaloid in *Cryptolepis*, has anti-plasmodial activity similar to that of chloroquine (Wright *et al.*, 1996). The mean IC_{50} value, determined from linear regression analysis of dose-response curves, was 0.114 micromolars for cryptolepine [1.11], compared to a mean value of 0.2 micromolars for chloroquine diphosphate. Cryptolepine [1.11] and its acid salts are known to be anti-malarial compounds that have the ability to inhibit the growth of *P.falciparum* (Boakye-Yiadom, 1979; Arzel *et al.*, 2001; Wright *et al.*, 2001).

Cryptolepine [1.11] has been found to possess several interesting pharmacological activities (Tackie and Schiff, 1993). The blood pressure lowering effect of the alkaloid cryptolepine [1.11] has been investigated and reported by (Noamesi and Bamgbose, 1980; Noamesi and Bamgbose, 1981; Oyekan et al., 1988; Oyekan and Okafor, 1989). It was found out that Cryptolepine [1.11] brought about dose dependent fall in blood pressure in both the systolic, diastolic and mean blood pressure changes were affected to the same extent by various concentrations of cryptolepine [1.11]. There have been extensive studies on the involvement of alpha-adrenoceptors mechanisms in the

hypotensive activity of cryptolepine [1.11] (Noamesi and Bamgbose, 1980). From the above research, it was indicated that cryptolepine [1.11] inhibits carrageenana-induced oedema in the rat hind paw sub-palter tissue. This was achieved by comparing the anti-inflammatory potency of cryptolepine with that of indomethacin and aspirin. In addition, Raymond-Hamet (Raymond-Hamet, 1938) observed that *in vitro* administration (5mg/kg) of the alkaloid produced a marked and protracted hypotensive response in the vagotomized dog with a corresponding disease in renal volume. These effects were apparently in response to the produced vasodilation produced by the alkaloid (Tackie *et al.*, 1993).

Cryptolepine [1.11] is believed to interact with Deoxyribonucleic acid (DNA) and this could result in toxicity (Kirby *et al.*, 1995). Evidence of DNA being the direct target of cryptolepine has been reported by Bonjean and his co-workers (Bonjean *et al.*, 1988). Cryptolepine [1.11] is also known to be a potent inhibitor of topoisomerase II. Its effect is to stop the cell from dividing and is probably the basis for its effect on microorganisms, including malaria parasite. It is also the basis for it being regarded as a promising anti-tumor agent (Bonjean *et al.*, 1998; Dassonneville *et al.*, 2000; Lisgarten *et al.*, 2002; Guittat *et al.*, 2003). It has however, been reported that extracts of cryptolepis used as tonic, can be taken daily for so many years without evidence of side effects or toxicity (Luo *et al.*, 1998).

Quindoline [1.12] isolated from the ethanolic and chloroformic leaf extracts and tested *in vitro* against *P. falciparum* gave 90% inhibition (Boakye-Yiadom, 1979; Cimanga *et al.*, 1996). Quindoline [1.12] has been shown to

possess activity against a number of cancer cells lines, including those that overexpress multidrug resistance (Opoku-Boahen, 1998). Quindoline [1.12] is known to be the least active anti-plasmodial of all the isolated compounds from *Cryptolepis sanguinolenta* (Noamesi *et al.*, 1983; Kirby *et al.*, 1995; Cimanga *et al.*, 1997). Although quindoline [1.12] is less active as an anti-malarial agent, its synthetic analogues are among the most potent anti-malarial drugs (Cimanga *et al.*, 1997). Biscryptolepine is known to exhibit only some activity against some Gram-positive bacteria (MIC = 62.5 or 31 µg/ml). The antibacterial activity of biscryptolepine is also bacteriostatic rather than bactericidal. (Cimanga *et al.*, 1996; Cimanga *et al.*, 1998)

Cryptospirolepine [1.13] a compound also isolated from the basic (alkaloid) fraction and obtained from the extract roots of *Cryptolepis sanguinolenta* has been shown to be responsible for the anti-malarial activity exhibited by the plant (Tackie and Schiff, 1993).

Cryptoheptine [1.14] also isolated from the *Cryptolepis sanguinolenta* is known to have anti-bacterial activity against gram-negative and Gram-positive bacteria (Paulo *et al.*, 1994).

Results have indicated that neocryptolepine shows an antibacterial activity against Gram-positive bacteria (MIC<100 µg/ml), but was less active against Gram-negative bacteria. It is also known to inhibit the growth of the yeast *Candida albicans*. It is also known that the anti-bacterial action of neocryptolepine [1.19] also isolated from the plant *Cryptolepis sanguinolenta* appears to mirror those of the major alkaloid, cryptolepine and it is bacteriostatic

rather than bactericidal (Cimanga *et al.*, 1998). Although initially neocryptolepine (also referred to as cryptotackieine) (Sharaf *et al.*, 1996) was reported to show an activity comparable to cryptolepine, more recent investigation have shown that it is about 7 times less active against the chloroquine-resistant *P. falciparum* Ghana-strain (Jonckers *et al.*, 2002). It is reported that 11-hydroxycryptolepine exhibits a more pronounced antiplasmodial activity than chloroquine when tested against the K-1 and W-2 strains (Cimanga *et al.*, 1997).

Isolation and Purification

The powdered roots are oven dried (40-60°C) and extracted with 80% ethanol to obtain the crude extract. These crude extracts are then taken through a classical acid/base extraction procedure for alkaloids using 10% HOAc and NH_4OH (pH= 9.5-10). Finally, the alkaline solution is extracted with $CHCl_3$ (5x 200ml) and the $CHCl_3$ extractives combined and dried over anhydrous Na_2SO_4, filtered and evaporated to afford a dark alkaloidal residue.

The alkaloidal residue is adsorbed on Al_2O_3 and chromatographed over Al_2O_3 with $CHCl_3$/ MeOH (different proportions) as the main elution solvents. Sometimes, a less polar petroleum-ether/$CHCl_3$ mixture is used followed by $CHCl_3$ and then $CHCl_3$-MeOH mixture.

At times, the powdered roots are first defatted with non-polar petroleum-ether prior to the ethanolic extraction. Methanol can also be used for the crude extraction instead of EtOH (Ablordeppey *et al.*, 1990).

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The extract is purified so as to isolate a purer extract or quindoline, cryptolepine or mixtures from the extract. Preferably, the extract is purified by ion exchange with a highly acidic resin such as DOWEX followed by high-performance liquid chromatography over a neutral polystyrene matrix, thereby isolating from the extract quindoline, cryptolepine or mixtures thereof.

Most preferably, the high-performance liquid chromatography column is eluted with water/acetonitrile/isopropanol. According to a preferred embodiment, reverse phase high performance liquid chromatography is performed using C-18 derivatized polymethacrylate (highly cross-linked). According to another embodiment, cross-linked polystyrene is used. As contemplated by the present invention, quindoline, cryptolepine or mixtures thereof may be used directly as hypoglycaemic agents.

In a preferred mode of extraction, the roots of *Cryptolepis sanguinolenta* are extracted with a solution of 1% acetic acid in water so described by Ablordeppey *et al.* (1990).

Colour Reaction of Indoloquinoline Alkaloids

Just like all alkaloids, quindoline alkaloids also react with alkaloid test reagents such as Dragendorff's, Mayer and Wagner's reagents to give coloured compounds. These characteristic colours are then used to ascertain the presence or absence of alkaloids in a given plant sample. Many colour reactions of alkaloids have been recorded in literature. One of the best-known test reagents is the Dragendorff's reagent, which gives an orange colour with most alkaloids.

Dragendorff's test reagent is made up of two solutions namely solution (A) and (B). The preparation of solution (A) involves dissolving a weighed amount of Bismuth nitrate in acetic acid and a measured amount of distilled water. The preparation of solution (B) also involves dissolving a weighed amount of Potassium iodide in a measured volume of distilled water. A mixture of equal volumes of solution (A) and (B) and some amount of distilled water produces the test reagent called Dragendorff.

The second test reagent that is commonly used to ascertain the presence of alkaloids is the test reagent known as Mayer's reagent that gives a yellowish precipitate in the presences of alkaloids. This test reagent is prepared by dissolving a weighed amount of mercuric chloride and potassium iodide in distilled water. Another test reagent used in alkaloid test is the Wagner's reagent. This test reagent gives a white coloured solution to indicate the presence of the alkaloid. The test reagent is prepared by dissolving a weighed amount of Iodine and Potassium iodide in an amount of distilled water.

Synthesis of Quindoline Alkaloids

Quindoline (also known as norcryptolepine) [1.12], was first described by Fichter and Boehringer in 1906 as a synthetic product (Fichter and Boehringer, 1988). The alkaloid was not isolated from a plant source until 1978, when Dwuma-Badu *et al.*, described the compound as a metabolite of *Cryptolepis sanguinolenta* (Dwuma-Badu *et al.*, 1978). Fichter and Boehringer synthesized quindoline [1.12] from bis-o-nitrobenzylmalonate [2.12] and sodium hydroxide to

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give the sodium salt of bis-o-nitrobenzylacetic acid. A boiling solution of the product with acetic acid gave dioxyquindoline [2.13] in 20% yield, which when treated with hydriodic acid and phosphorus followed by sodium hydroxide gave quindoline [1.12] (Fig.2.0).

[2.12]

[2.13]　　　　　　　　　　　[1.12]
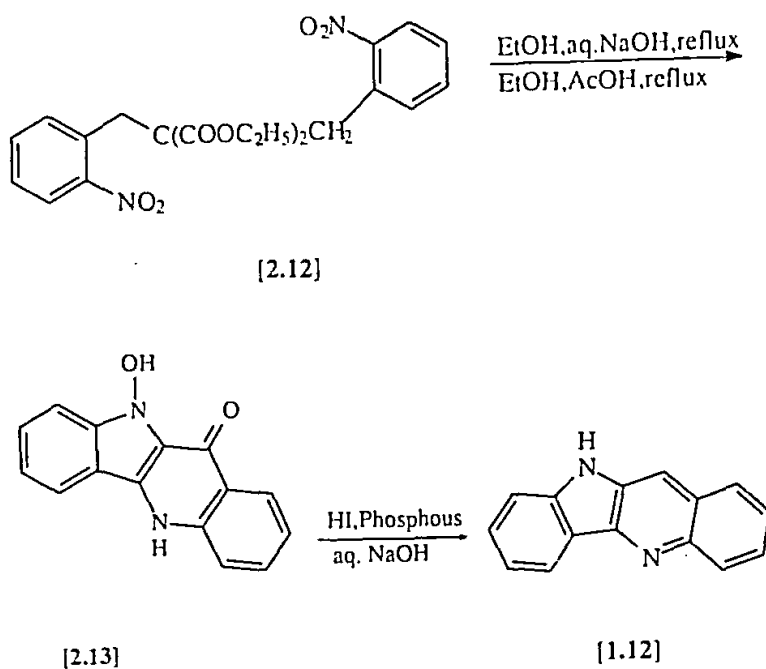
Fig. 2: Synthesis of quindoline from bis-o-nitrobenzylmalonate

In their procedure, Holt and Petrow synthesized quindoline-11-carboxylic acid [2.16] by condensation of O, N-diacetylindoxyl [2.14] and isatin [2.15]. Decarboxylation of the acid [2.16] gave quindoline [1.12]. The method was basically the Pfitzinger quindoline synthesis in which the O, N-diacetylindoxyl [2.14] has replaced the ketone (Holt and Petrow, 1947).

[2.14]   [2.15]

[2.16]   [1.12]
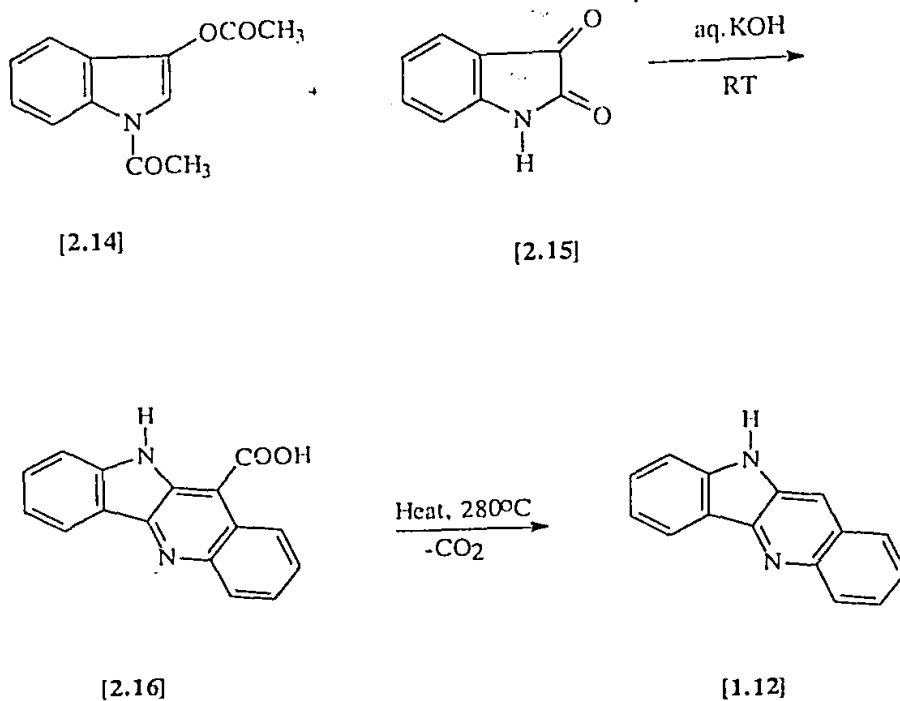
Fig. 3: Synthesis of quindoline using O, N-diacetylindoxyl and isatin

Since then, many researchers have also used various methods to synthesize quindoline from various starting materials (Opoku-Boahen, 1998).

One of such methods is the utilization of intramolecular β-Nucleophilic Substitution of a 1-phenylsulfonyl-2-acylindole.

Quindoline (=des-N-methylcryptolepine) was obtained from cryptolepine by selenium dehydrogenation; conversely, N-methylation of quindoline gave the hydriodide of cryptolepine (Gellért et al., 1951).

Fig. 4: β-Nucleophilic substitution of a 1-phenylsulfonyl-2-acylindole

22
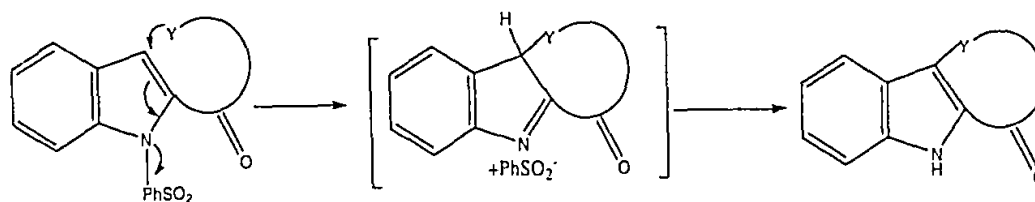
Melanie *et al.* (1996) have previously demonstrated the above process in their work. It is an operation of a process (Fig. 4.0) of overall intramolecular nucleophilic substitution at an indole β-position, where the indole carries a phenylsulfonyl group on nitrogen and a ketonic substituent at C-2; the N-substituent is expelled as phenylsulfinate; the process may be synchronous (Fig. 4.0), or may involve an intermediate in which the nucleophile has added to the formal enone unit. In the examples so far described (Dalton *et al.*, 1983) the nucelophilic centre (Y) was alcoholic oxygen.

In an earlier work by Melanie, *et al*, they utilized the condensation product from 2-lithiated 1-phenylsulfonylindole (Sundberg *et al.*, 1973) and phthalide to illustrate the operation of the indole- β-nucleophilic substitution process producing thereby a [1.12] benzoxepino[4,3-b]indole (Cooper *et al.*,1981; Glendinning *et al.*,1993) Extrapolating this to a nitrogen equivalent required benzoylphthalimidine which was prepared from phthalimidine (Melanie *et al.*, 1996) by reaction with benzoyl chloride in N,N-dimethylaniline. Reaction with 2-lithio-1-phenylsulfonylindole took place at both carbonyl groups generating a mixture of desired ketone [2.17] together with 2-benzoyl-1-phenylsulfonylindole, in a ratio of 4:3, separated by chromatography.
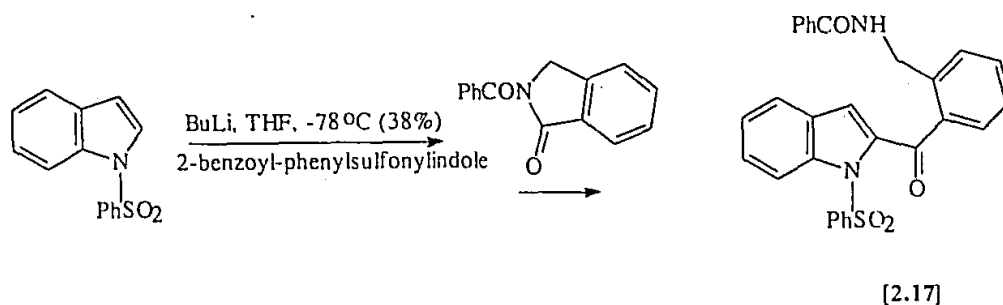
Fig. 5: Reaction leading to the formation of the desired ketone

If the reaction was allowed to proceed for a longer than ca.15 min a different, yellow product [2.18] was also formed which proved to be the result of the desired intramolecular nucleophilic substitution by the anion of the amide, and could be obtained from ketone [2.17] in good yield on exposure to NaH in refluxing THF. The corresponding amino-ketone [2.19] was readily obtained by alkaline hydrolysis of [2.18] (Fig. 6.0).
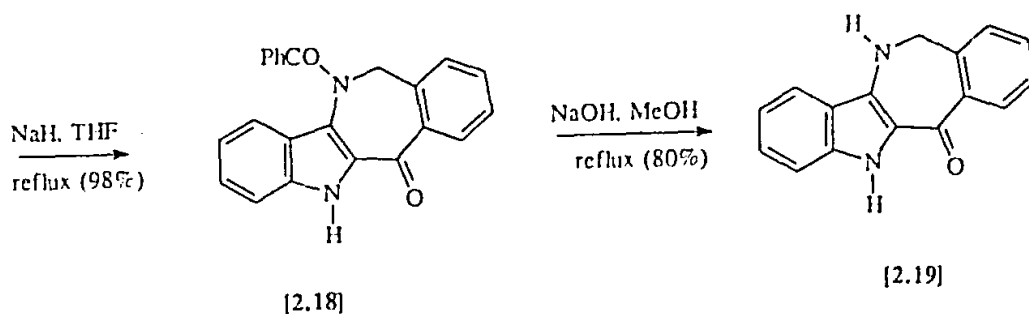
Fig. 6: Alkaline hydrolysis to yield the indole alkaloid [2.19]

Addition of lithiated 1- phenylsulfonylindole to 2-nitrobenzaldehyde followed by MnO₂ oxidation of the alcohol, catalytic reduction of the nitro group and N-benzoylation gave amido-ketone [2.20]. N-Deprotonation using NaH allowed ring closure in hot THF to tetracycle [2.21], hydrolysis of which produced [2.22].
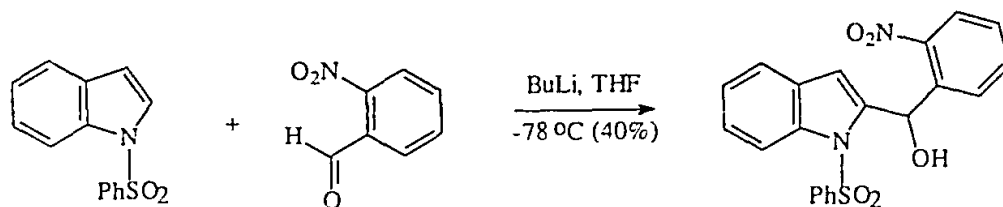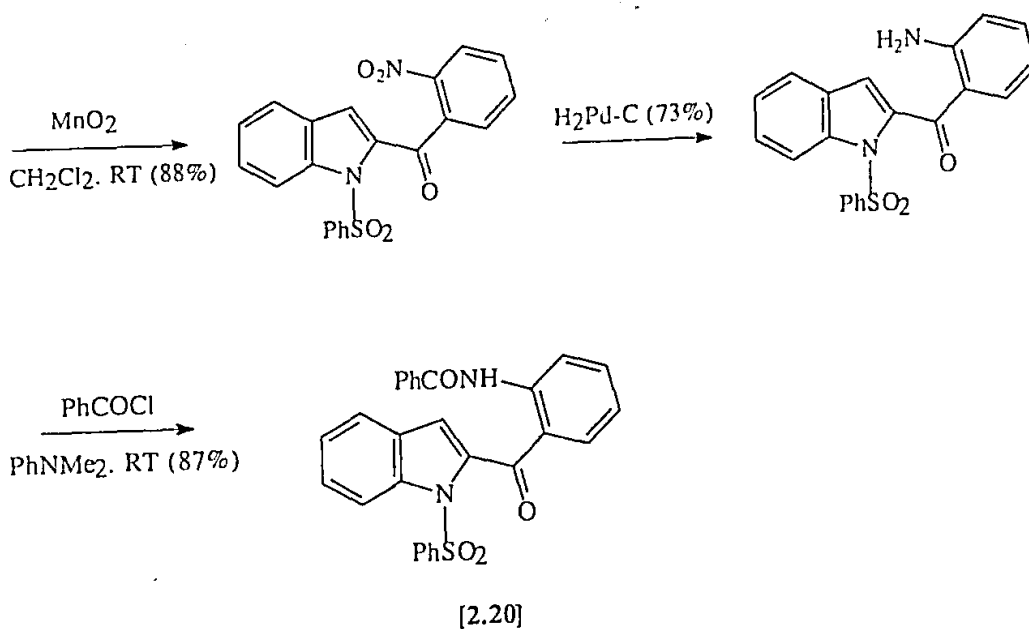
Fig. 7: The synthesis of an amido-ketone

N-methylation of the quinolone [2.22] with NaH as base and at room temperature produced crystalline material to which was ascribed the structure [2.23] and which had electronic absorption and tlc behaviour identical with those of hydroxycryptolepine (Paulo *et al.*, 1995) The carbonyl tautomeric forms of [2.22] and [2.23], shown, are established by spectroscopic comparisons. Thus UV/VIS absorptions of [2.22] and [2.23] and of the alkaloid, (Paulo *et al.*, 1995) in neutral and acidic solution were identical; only [2.22] showed a significant change in alkaline solution, no doubt due to deprotonation at the quinolone N-hydrogen (Spitzer *et al.*, 1991).

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NaH, THF
reflux (80%)

[2.21]

NaOH, MeOH,
heat (85%)

[2.22]

NaH, MeI, DMF, RT (30%)

[2.23]
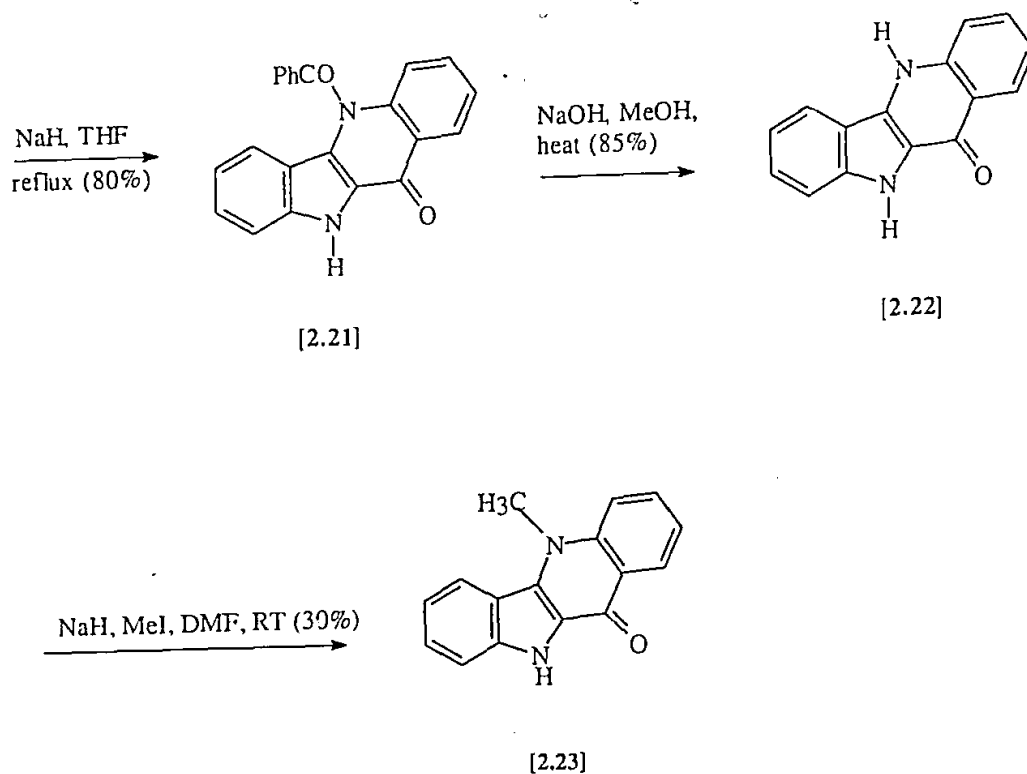
Fig. 8: Synthesis of a quindoline derivative

Treatment of [2.21] with NaBH$_4$ in refluxing ethanol did not effect the expected reduction of the carbonyl group. However heating [2.24] in POCl$_3$ produced [2.25], catalytic hydrogenolysis of which gave quindoline (Fig.9.0).

[2.24]

POCl, reflux (95%)

[2.25]

26
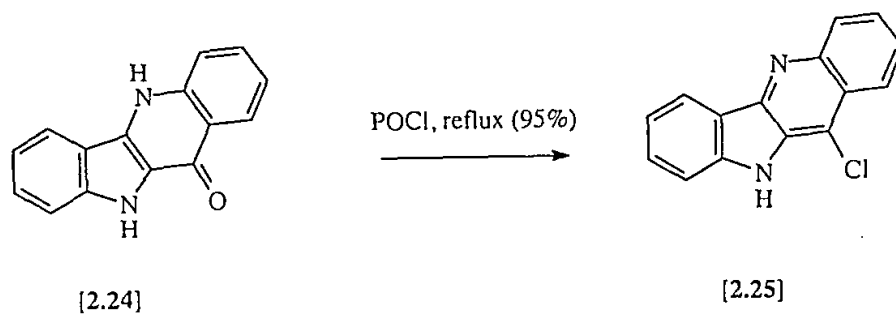
H₂,Pd-C, EtOH, (95%)

$$H_2, Pd\text{-}C, EtOH, (95\%)$$
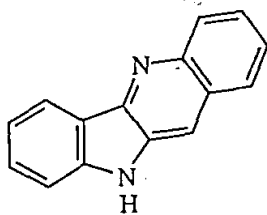
[1.12]

Fig. 9: Synthesis of quindoline from a carbonyl compound

Currently, considerable interest has arisen for the synthesis of indoloquinoline alkaloid derivatives, due to their potential biomedical and pharmaceutical values. Using various synthetic routes, a number of substituted cryptolepine analogues have been made and evaluated for their potential as leads to new anti-malarial agents, antitumor agents etc.

The compound (quindoline alkaloid) whose skeleton contains the quinoline fragment has been the subject of much interest in synthetic organic chemistry, since many of them reveal strong cytotoxic and antitumor activity combined with relatively low toxicity (Kamienska-Trela *et al.*, 1995).

The following groups of quindoline derived compounds (2.26 and 2.27) have been reported in an earlier work by Yamato *et al.* (1990).

27

[2.26]

[2.27]
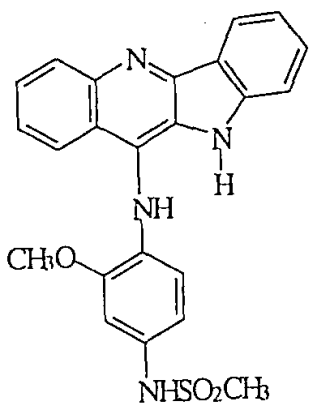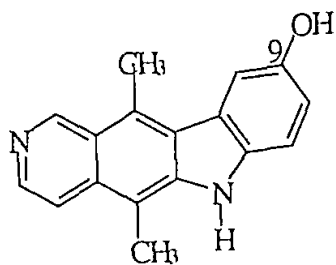
9-hydroxyellipticine

Fig. 10: Examples of quindoline derived compounds

The 4-methoxyindolo[3,2-b]quinoline [2.26a] and 4-hydroxyindolo[3,2-b]quinoline [2.26b] derivatives were prepared from 3-methoxy-2-[(N-phenylamino) acetamido]benzoic acid [2.30] via the key intermediate 11-chloro-4-methoxyindolo[3,2-b]quinoline [2.32] (Fig.13). The intermediate [2.30] was obtained by the chloroacetylation of 2-amino-3-methoxybenzoic acid [2.28] followed by amination with aniline [2.29a].

[2.28]

[2.29]
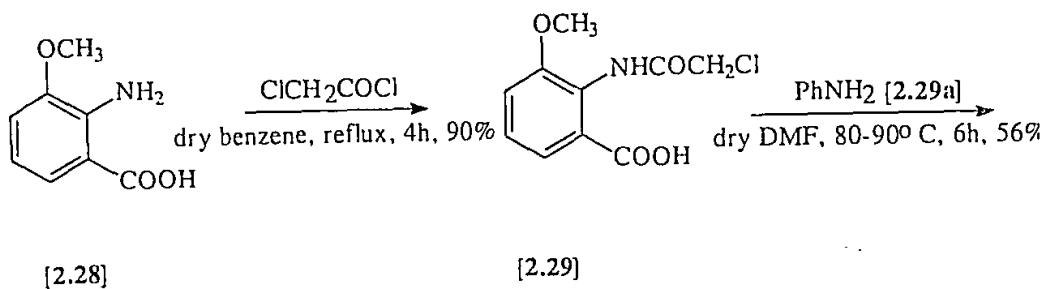
Fig. 11: Chloroacetylation of 2-amino-3-methoxybenzoic acid

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The intermediate [2.31] was prepared through the intramolecular condensation of [2.30] by heating with polyphosphoric acid (PPA). Treatment of [2.31] with phosphorus oxychloride (POCl₃) gave [2.32]. The demethylation of [2.32] by heating with 47% hydrobromic acid gave 11-chloro-4-hydroxyindolo [3,2-b]quinoline derivative [2.33].
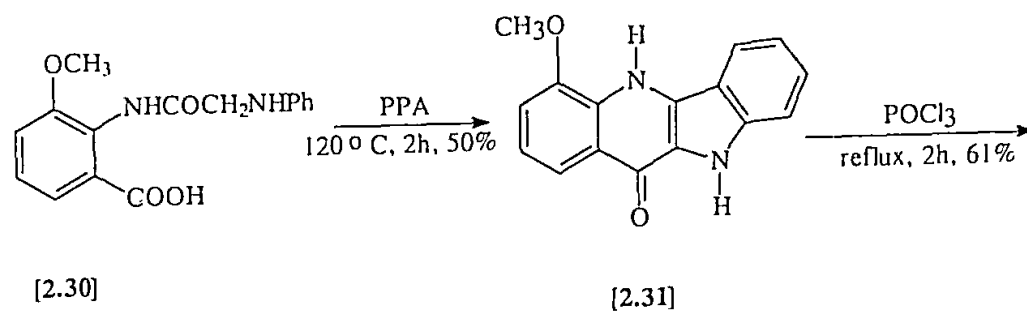
Fig. 12: Intramolecular condensation

Amination of [2.32] and [2.33] with N-(4-amino-2-methoxyphenyl) methanesulfonamide hydrochloride [2.32a] (Cain et al., 1975) successfully afforded [2.26a] and [2.26b], respectively.
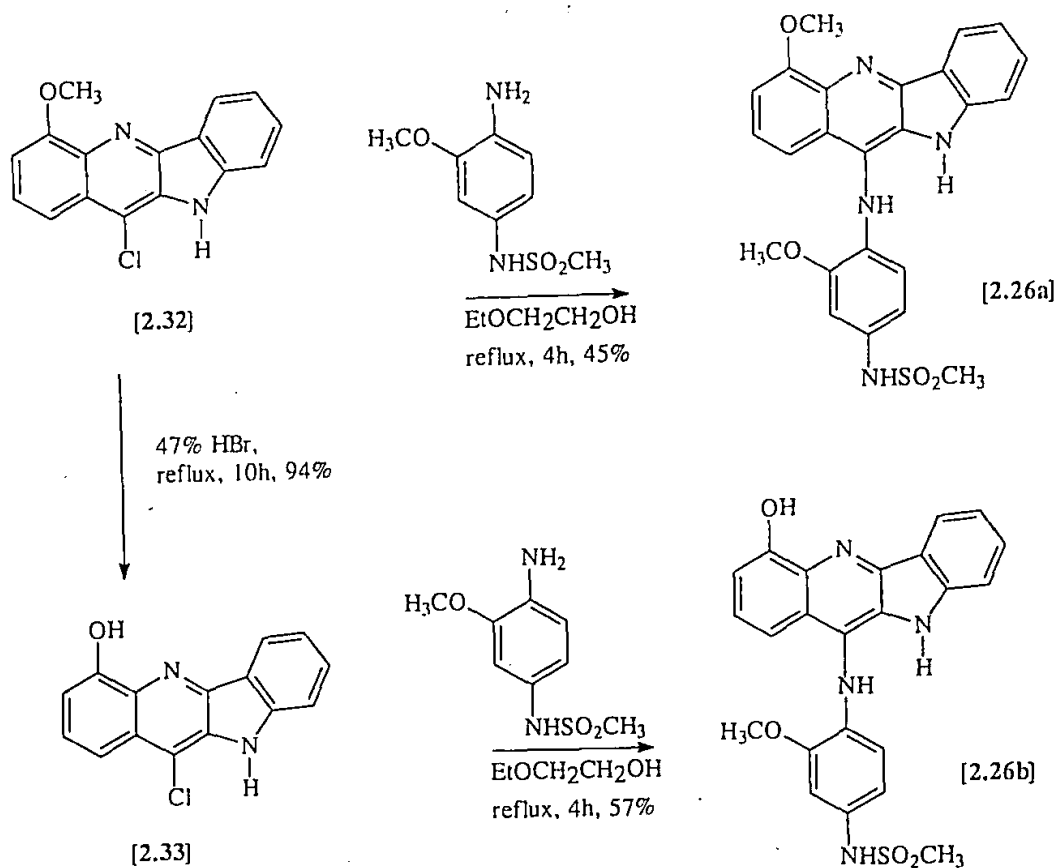
Fig. 13: Synthesis of quindoline derived compounds

In the preparation of [2.26c] and [2.26d], the key intermediate, 7-methoxy-5H, 10H-indolo[3,2-b]quinoline-11-one [3.37]could not be obtained by the same procedure for the synthesis of [2.22]. Namely, treatment of 2-{[N-(4-methoxy-phenyl)amino]acetamido}benzoic acid [2.35] with PPA or polyphosphoric acid ethyl ester (PPE) gave only water-soluble inseparable products. Therefore, N-protected congeners of [2.35] introduced by an acetyl, tosyl, benzyl, or 3,4-dimethoxybenzyl groups to the nitrogen atom of aniline moiety [2.36] were synthesized and were attempted to give a corresponding

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derivative of [2.37] by the same procedure for the synthesis of [2.31]. Among these examinations, only the N-benzyl derivative [2.31] afforded the desired intermediate [2.37] with loss of the benzyl group.
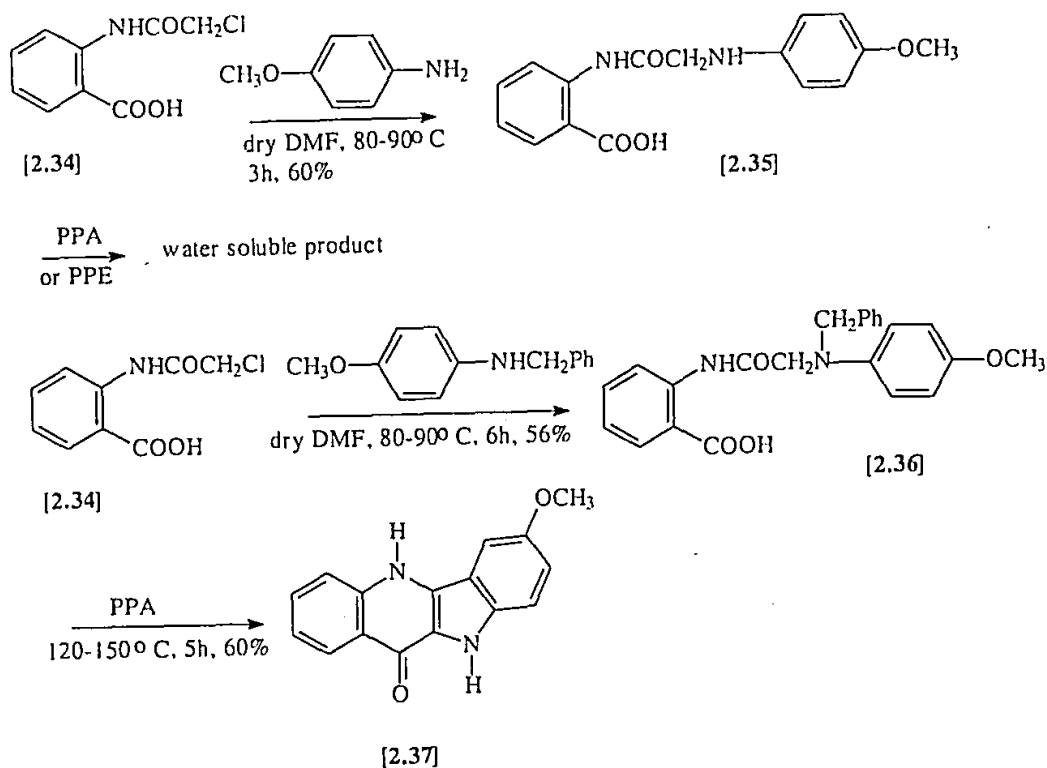
Fig. 14: An intermediate leading to the synthesis of quindoline

Other congeners gave but water-insoluble inseparable products. Compounds [2.26c] and [2.26d] were prepared from [2.28] according to the similar method for the preparation of [2.26c] and [2.26d], respectively.

31
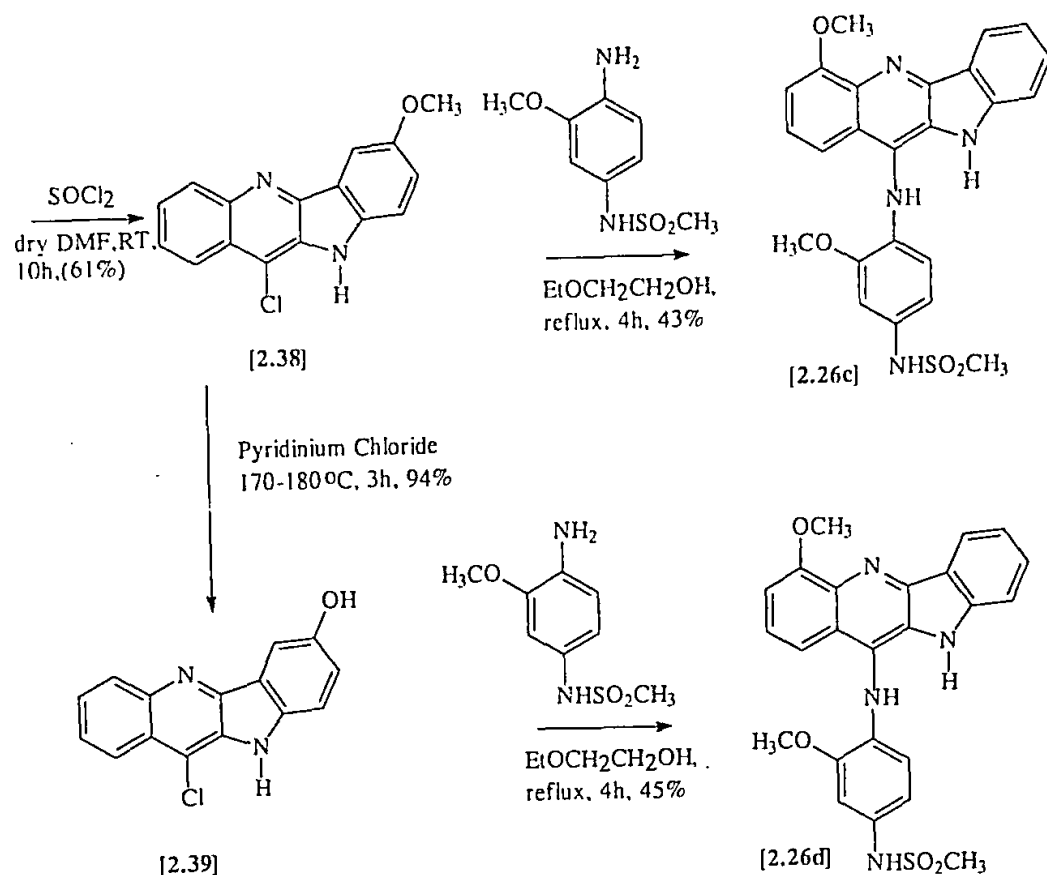
Fig. 15: Synthesis of quindoline derived compounds

Spectral Properties of Quindoline Alkaloids

In this review, an attempt is made to present in summary form some spectroscopic methods employed in the structure elucidation of quindoline alkaloids.

Ultra violet Spectral Properties of Quindoline Alkaloids

UV: λmax (methanol) 227 (log ϵ 4.30), (quinoline) 269sh (4.45); 274(4.46); 330sh (3.82), and 345(4.03) nm;

32
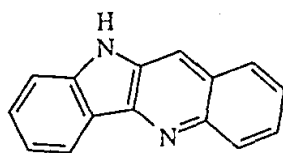
[1.12]

Infrared Spectral Properties of Quindoline Alkaloids

Infrared spectral data have been invaluable in the investigation of virtually all classes of alkaloids especially in the detection of functional groups. In the alkaloids, as in any other class of Organic compounds, the appearance or absence of certain key bands in an IR spectrum is more or less certain indication of the presence or absence of the particular groups with which they are associated.

IR: νmax (nujolmull)/cm^{-1} 3489(N-H), 1632, 1608(C=C), 1487, 1396, 837, 813, 754, 710(aromatic)

^{1}H and ^{13}C-NMR of Quindoline Alkaloids

NMR Spectroscopy has been used to detect and identify double bonds, to determine the nature of end groups and also used to ascertain the origin of methyl groups in the molecule. In certain cases, definite structures have been assigned on the basis of NMR spectra.

^{1}H-NMR, it helps to identify the carbon-hydrogen framework of an organic compound. Protons in exactly the same environment with respect to the rest of the molecule are called chemically equivalent protons. Each set of chemically equivalent protons in a compound gives rise to a signal (resonance) in the NMR spectrum of that compound. The number of signals in a ^{13}C-NMR

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spectrum tells us how many different kinds of carbons a compound has, just as the number of signals in ^{1}H-NMR spectrum tells us how many different kinds of protons a compound has.

The circulations of delocalized π electrons generate magnetic fields that can either shield or deshield nearby protons. Whether shielding or deshielding occurs depends on the location of the proton in the induced field. The aromatic protons of benzene derivatives are deshielded because their locations are such that the induced magnetic field reinforces the applied magnetic field. Because, of this deshielding effect, the absorption of energy by aromatic protons occurs downfield at relatively low magnetic field strength.

In ^{13}C-NMR spectrum, the chemical shifts range over about 220ppm compared to about 10ppm for ^{1}H-NMR. Carbons close to electron-rich environment produce upfield signal and carbons close to electron-withdrawing groups produce downfield signals.

Beginning from the 8.27 ppm singlet, couplings were observed to the carbons resonating at 145.7, 143.4, and 127.4 ppm. The first two resonances of this group were quaternary carbon resonances, the last was the protonated carbon bound directly to the proton resonating at 8.09 ppm. The proton resonating at 8.09 ppm was also long-range coupled to the quaternary carbon resonating at 143.4 ppm, as was the proton meta to it at 7.64 ppm. Hence, the carbon resonating at 143.4 ppm is assigned as C4a. The other quaternary carbon resonating at 145.7 ppm must thus be the C5a resonance. Further confirmation of the assignment of C5a is afforded by the long-range coupling of the 145.7 ppm resonance to the NH

resonance at 11.4 ppm. The latter assignment is also confirmed by the weak three-bond connectivity from the proton resonating at 8.35 ppm, which must be the H7 resonance. A summary of all of the long-range connectivities to the quaternary carbons is shown below (Spitzer *et al.*, 1991).
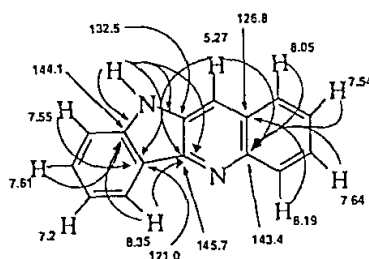
The carbon resonating at 144.1 is assigned as C9a and is long-range coupled with protons at 8.35 and 7.61 ppm, which are assigned as H6 and H8 respectively, as well as the NH resonance at 11.4 ppm. The carbon resonating at 121.0 ppm is long-range coupled to protons resonating at 7.55 and 7.27 ppm (H7 and H9, respectively) and may thus be assigned as C5b. A coupling to the C5b resonance from NH resonance at 11.4 ppm is also observed. The carbon resonating at 126.8 ppm is long-range coupled to protons resonating 7.54 and 8.19 ppm (H2 and H4, respectively) and may be assigned as C4a. Finally the quaternary carbon resonating at 132.5 ppm exhibits only a long-range coupling in the HMBC spectrum, that to the NH proton (Spitzer *et al.*, 1991).

A total assignment of the ^{1}H and ^{13}C-NMR spectra, in DMSO-d6 has been carried out by Martin *et al.* (2002) as part of extensive studies of the spectra of the compound, isolated from the root of the medicinal plant *Cryptolepis sanguinolenta* (Spitzer *et al.*, 1991).

Table 1: Protons and Carbon NMR Resonance Assignments for 10H-indolo[3,2-b]quinoline (Spitzer et al., 1991).

Position	δ^{1}H (ppm), M, J/Hz		δ^{13}C (ppm).
			145.7
5a	-		144.1
9a	-		143.2
4a	-		132.5
10a	-		129.8
8	7.61	dd, 8.3, 7.3	128.8
4	8.19	d, 8.3	127.6
1	8.09	d, 8.1	126.8
11a	-	- -	126.1
3	7.64	dd, 8.5, 6.7	124.9
2	7.54	dd, 8.1, 6.6, 12	121.4
6	8.35	d, 8.4	121.0
5b	-	- -	119.4
7	7.27	dd, 8.4, 7.3	113.1
11	8.27	s -	111.58
9	7.55	d, 8.3	-
NH	11.4	-	

[1.12]

Mass Spectral Properties of Quindoline Alkaloids

Mass spectroscopy is now being increasingly used as a means of elucidating the structure of most compounds. Thus, it is possible to determine

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molecular weights, molecular formulae, the nature of various functional groups, and the relative positions of double bonds.

The mass spectrum of compound [1.12] shows a molecular ion at m/z 218 (100%), 217(8), 190(8), 109(12), 95.5(4) 90(3), and 89(5). which is found to be consistent with the molecular formula $C_{15}H_{10}N_2$.
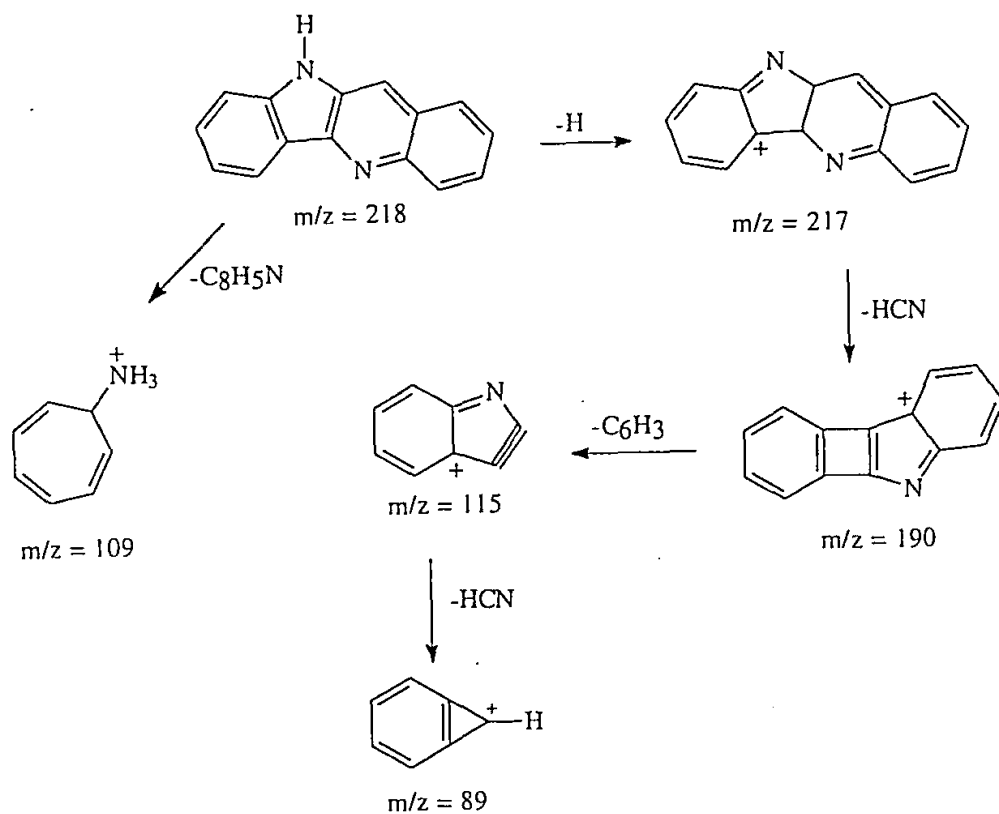
Fig.16: Fragmentation pattern of the compound quindoline

CHAPTER THREE

EXPERIMENTAL

Collection and Identification of Plant Material

The sample stem (bark and wood) of the plant *Cryptolepis sanguinolenta* was collected from the Centre for Scientific Research into Plant Medicine, Mampong-Akwapim, in the eastern region of Ghana. It was examined and identified by Dr Yaw Ameyaw a Botanist at the Centre for Scientific Research into Plant Medicine. This was however, corroborated by Mr Agyakwah at the Botany Department of the University of Cape Coast.

Treatment and Screening of the Plant Material

The stem was thoroughly washed with water and then chopped into small pieces. It was dried under the sun for two weeks and milled into powder. The total weight of this powder was recorded to be 461.5g.

The dried and powdered stem of *Cryptolepis sanguinolenta* was extracted successively (see flow sheet -1) to obtain a number of extract residues.

Thus the powdered stem was extracted repeatedly with petroleum ether (40-60°C) in a continuous soxhlet extractor until further extraction gave a colourless solution. The yellowish-brown extract was concentrated and evaporated to give a dark brownish yellow paste. The defatted stem was

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completely freed of last traces of solvent by leaving in the air. The marc was then exhaustively extracted with methanol (95%) and concentrated to dryness. The crude methanol extract (30.0g) was dissolved in 10% acetic acid and further diluted with distilled water. The crude extract solution was then filtered to remove the insoluble non-alkaloidal components. The insoluble components were exhaustively washed with more of the 10% acetic acid and left overnight. It was then filtered under gravity and the residue discarded. The acetic acid extract was basified with aqueous ammonia to a pH of 9. It was then shaken three times with chloroform in a large separatory funnel and the chloroform extract evaporated.

This general extraction methodology was considered ideal for the isolation of most chemical constituents that occur in plants. This was necessary, as the medicinal value of the plant requires such a screening procedure.

completely freed of last traces of solvent by leaving in the air. The marc was then exhaustively extracted with methanol (95%) and concentrated to dryness. The crude methanol extract (30.0g) was dissolved in 10% acetic acid and further diluted with distilled water. The crude extract solution was then filtered to remove the insoluble non-alkaloidal components. The insoluble components were exhaustively washed with more of the 10% acetic acid and left overnight. It was then filtered under gravity and the residue discarded. The acetic acid extract was basified with aqueous ammonia to a pH of 9. It was then shaken three times with chloroform in a large separatory funnel and the chloroform extract evaporated.

This general extraction methodology was considered ideal for the isolation of most chemical constituents that occur in plants. This was necessary, as the medicinal value of the plant requires such a screening procedure.

Flow Sheet – 1

General Extraction Procedure
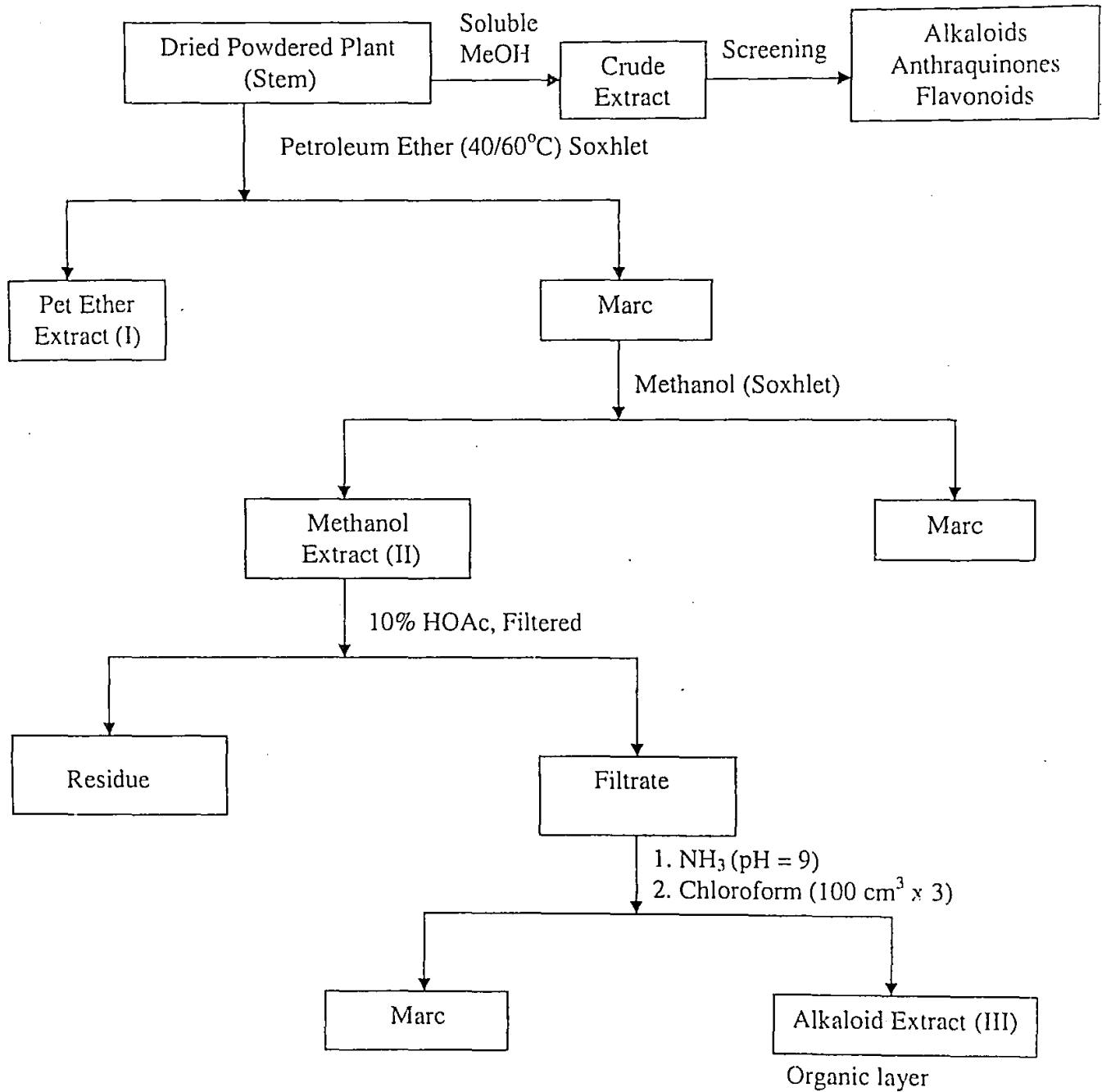
Fig.17: Flow chart of the General Extraction Procedure

Investigation of Extracts

The petroleum ether extract (I) (see flow sheet – 1) from the extraction of petroleum ether was kept in the dessicator and no work was done on it.

In order to determine the number of components present in the sample, the methanol extract was subjected to thin layer chromatography. It was discovered that alumina was the best adsorbent for separating the components. The solvent system, chloroform/methanol (95:5) resolved best the components of extract (II). The plates were observed under ultraviolet lamp (**Fig. 18**).

The chloroform extract (Alkaloid) was chromatographed on alumina. Chloroform and methanol mixtures were mainly used for elution. In all, hundred fractions were collected, concentrated and further examined on thin layer chromatogram.

The methanol extract (II) showed seven main spots on silica gel chromatogram using chloroform-methanol (95:5) as the mobile phase (**Fig. 18**). Further separation into pure compounds was done on preparative thin layer chromatography plates.
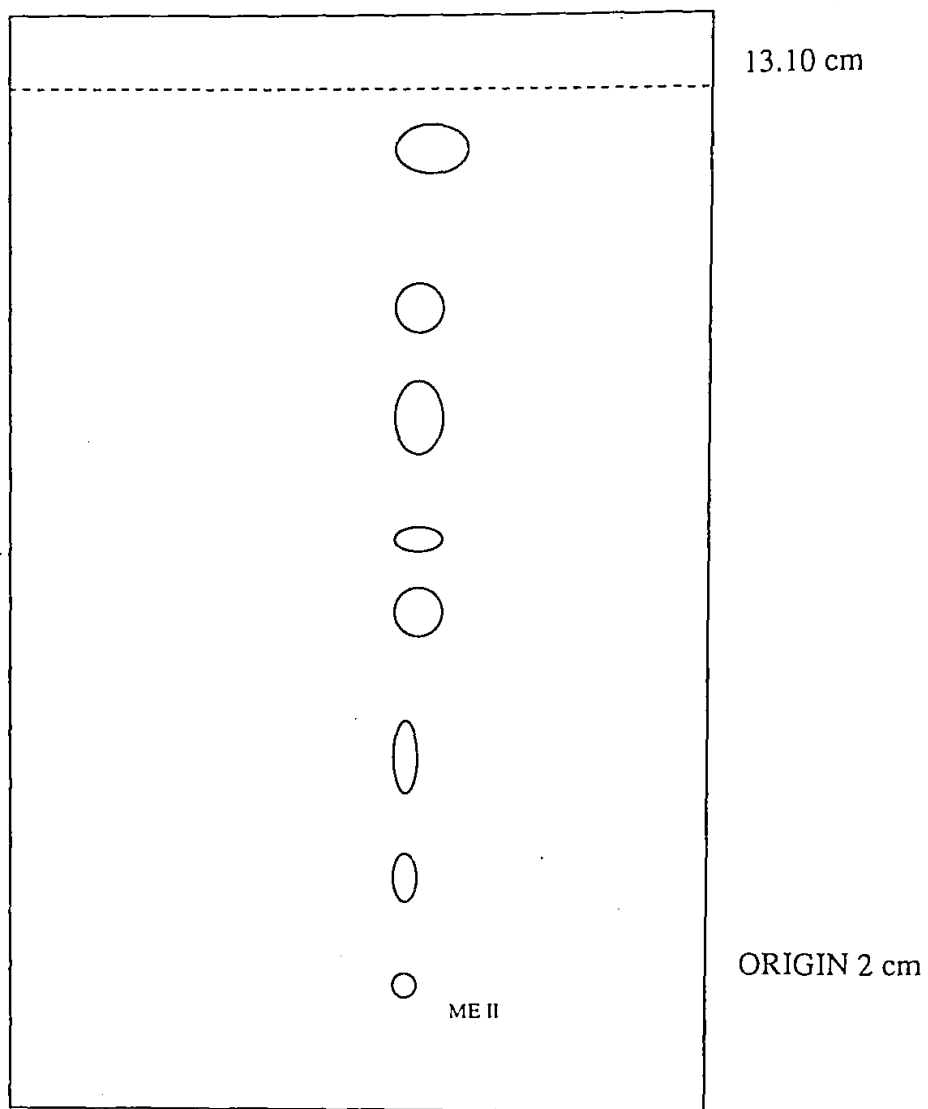
13.10 cm

ORIGIN 2 cm

ME II

Fig. 18: Thin Layer Chromatogram of Methanol Extract (II) on Silica Gel.

Solvent: -Chloroform: Methanol (95:5)

```
COLOUR UNDER                    Rf
UV LAMP
- - - - - - - - - - - - - - - - - - - - - - -        7.3 cm

Bright yellow    ()      CH4     0.6


Pale yellow      ()      CH3     0.4



Violet           ()      CH2     0.3



White oily liquid ()     CH1     0.2


                  ⬯   AE III          ORIGIN 2 cm
```

Fig. 19: Thin Layer Chromatogram of Isolated compounds (IV) on Silica Gel. Solvent: - Chloroform

Detection of Alkaloids

(a).   Dragendorff's Test (Farnsworth *et al.*, 1977)

A filter paper was impregnated with a few drops of the ethanol extract and dried. Few drops of Dragendorff's reagent were added to the impregnated dried filter paper. The Dragendorff's reagent was washed with distilled water in a dropwise manner. There was a persistence appearance of orange colour, which showed the presence of alkaloids.

(b). Mayer's and Wagner's Test

A volume of 100 cm^3 of 60% ethanol extract was measured into an evaporating dish and evaporated to a syrupy mass consistency over a steam bath. 10.0 cm^3 of 2M HCl was added to the syrup on the steam bath and stirred for 3 minutes and then cooled to room temperature. 1.0g of sodium chloride was added, stirred and filtered. The residues were washed with sufficient volume of additional HCl to bring to the final volume of 10.0 cm^3.

The clear filtrate was divided into four equal parts in separate test tubes. To the first and second test tubes were added Mayer and Wagner's reagent respectively. These two test tubes were observed alongside with the third and fourth test tubes. There was the occurrence of a yellow-white precipitate in these test tubes. This indicated the presence of alkaloids.

Table 2: Alkaloid Test Results.

Wt. Extracted	Mayer's	Dragendorff's	Wagner's	10% Tannic acid
70g	+++	+++	+++	+++

Key: + = Precipitate

*Refer to appendix for details of test reagents.

Confirmatory Test of Alkaloids

The two remaining portions of the filtrate above were combined and four drops of concentrated ammonia (NH$_3$) was added to render the filtrate alkaline.

After shaking the filtrate, it changed red litmus paper to blue. The alkaline solution was then transferred into a small cleaned separatory funnel and extracted with chloroform (3 x 10 cm^3). The three chloroform extracts were combined into a small evaporating dish and evaporated to dryness using the water bath. 5.0 cm^3 of 2N HCl was added to the residue and stirred for 2 minutes. It was cooled to room temperature and filtered into a test tube. 0.25g of powdered sodium chloride (salting out agent) was added, stirred and filtered into a test tube. The residue was washed with sufficient volume of the 2N HCl to bring the volume to 10 cm^3. The clear filtrate was transferred into 5 test tubes (2 cm^3 each) the filtrate in the test tubes were then screened for alkaloids by adding few drops of each of Mayer's reagent, Dragendorff's reagent, 10% tannic acid, and Wagner's reagent to each of the 2 cm^3 portions in the 5 test tubes. They were immediately observed, and further observed after 5 minutes. The results obtained confirmed the presence of alkaloids. These tests were performed on the isolated alkaloids.

Table 3: Alkaloid Confirmatory Test Results.

Wt. Extracted	Mayer's	Dragendorff's	Wagner's	10% Tannic acid
70g	+++	+++	+++	+++
	Yellowish ppt	Cloudy orange ppt	Whitish brown	White ppt

Key: + = precipitate *Refer to appendix for details of test reagents

From the table of result (table 2 & 3), it was evident that alkaloids are present in the plant sample.

Detection of Anthraquinone

Borntrager Reactions (Farnsworth *et al.*, 1977)

The powdered sample (0.3g) was extracted with (80%) ethanol solution. The ethanol extract was evaporated in a crucible over a water bath to dryness. The residue was then re-dissolved in 30 cm^3 of distilled water and filtered. The filtrate was then shaken with benzene (10 cm^3) (takes on light yellow colour) in a separator. The sample (5 cm^3) of this benzene extract was shaken with ammonia test solution (2.5 cm^3). There was no red colour in the alkaline layer and hence an indication of the absence of anthraquinone.

Detection of Flavonoids

4 cm^3 of the ethanol extract was measured into a crucible and evaporated to dryness on a water bath. The residue was dissolved in 15 cm^3 of petroleum ether and filtered. The defatted residue was dissolved in 30 cm^3 of 80% ethanol and filtered. 2 cm^3 of the filtrate was measured into 2 test tubes. The test was performed using 0.5 cm^3 of concentrated HCl and 3 pieces of magnesium turnings (ribbon) were added. The second test tube served as the control and nothing was added to it. There was no colour change in the first test tube different from the blank. This indicated the absence of Flavonoids.

Concentration of Extracts

The samples extracted were concentrated and evaporated under vacuum at temperature of 50°C using the rotary evaporator. The samples were kept in the dessicator over activated silica gel.

Screening of the Stem for Constituents

About 461.5g of the powdered stem was defatted by exhaustive extraction with 2000 cm^3 petroleum ether (40-60°C) in a soxhlet extractor. The petroleum ether extract was evaporated to give 5.60g of sample. It was next exhaustively extracted with methanol (95%) and concentrated to dryness. The crude methanol extract 30.0g was dissolved in 1500 cm^3 of 10% acetic acid and diluted with 500 cm^3 of distilled water. The solution was filtered to remove the insoluble non alkaloidal components. The insoluble components were exhaustively washed with 500 cm^3 of the 10% acetic acid and left overnight. It was then filtered and the residue discarded. The acid solution was basified (pH = 9) with ammonia at room temperature and extracted to exhaustion with chloroform. The solution was shaken three times with chloroform in a large separatory funnel. The chloroform layer (organic) was dried with anhydrous magnesium sulphate and evaporated to dryness to give 4.7g of residue (alkaloidal component).

Thin Layer Chromatography (Bobbit, 1964)

The technique of thin layer chromatography (TLC) used was for resolving the crude extracts into its components and for checking the purity of any compound that could be isolated. In addition, it was used to select the best solvent system of separation and isolation. Differing polarity and ratios of various solvents were employed in this attempt to arrive at the best solvent system. It was essentially qualitative.

The thin layer of adsorbent used for the TLC were supported and held in place by glass plates. The glass plate sizes used were 5 x 20 cm, 10 x 20 cm and 20 x 20 cm. The plates were immersed in chromic acid to remove all grease. The plates were then washed with detergent and water and dried in the oven. The dried plates were assembled on their support and finally wiped with cotton wool moistened with some acetone.

Thirty grams of silica gel G (type 60 Merck) incorporating plaster of Paris binder were weighed into a beaker. Sixty cubic centimetres of water was added and the mixture stirred vigorously for about a minute. The slurry was quickly put into the spreader placed at one end of the plates and then spread over the plates in the usual way. The plates were left to dry at room temperature.

Before use, the plates were baked in the oven at 110°C for one hour and stored in a dessicator over silica gel. Bands of film approximately 5 mm wide were removed from the edges of the plates. The origin was usually at a point 2.0 cm from one edge of the film. The upper limit, which was 10 – 15 cm from the origin, was marked.

The sample dissolved in a suitable solvent was released onto the origin by touching the tip of a filled capillary to the adsorbent layer with the help of a spotting template and then allowed to dry. The plate was then placed in a developing tank lined with sheets of filter paper to ensure homogeneity of the atmosphere. The following solvent systems were tried as shown in table 4 below.

Table 4: Solvent system

Solvent system	Ratio/State
(i) Petroleum ether (40-60°C)	pure
(ii) Chloroform	pure
(iii) Methanol	pure
(iv) Petroleum ether (40-60°C)/Chloroform	(1:1)
(v) Chloroform /Methanol	(95:5)
(vi) Chloroform/Methanol	(9:1)
(vii) Petroleum ether (40-60 °C)/Methanol	(4:1)

The volumes of solvent were measured separately in measuring cylinder and then poured into the developing tank at least thirty minutes before development was started. This was done in order to achieve equilibrium between the gaseous phase and the liquid phases. After development the plates were dried in air for about ten minutes. The spots were located on the plates by the combination of the following methods:

(a) Examining under ultraviolet lamp (UV 254 x 366)

(b) The plates were exposed to iodine vapour.

Column Chromatography (Stock, *et al.*, 1963)

A column 60 cm long with an internal diameter of 3 cm was used. The adsorbent (alumina) was activated at 110°C for two hours and kept in the dessicator before use.

The column was washed with chromic acid, detergent, then water and finally with acetone and dried. It was filled with the solvent to be used initially for elution and then a small bit of cotton wool to serve as a support. The alumina was made into slurry with the solvent and poured into the column with gentle tapping until the desired column height was obtained. 100g of alumina was used for each gram of mixture to be separated. A small bit of cotton wool was again placed at the top of the column to form a protective band.

The mixture (4.7g) was dissolved in chloroform and about 2g of alumina was added. This was stirred vigorously on a steam bath until it turned powdery. The dry powder was then introduced onto the top of the column. The following solvent systems were used in the order given in Table 5 below.

Table 5: Solvent systems

Solvent system	Ratio/State	Quantity
(i) Petroleum ether (40-60 °C)		1000 cm^3
(ii) Petroleum ether (40-60 °C): Chloroform	(4:1)	500 cm^3
(iii) Petroleum ether (40-60 °C): Chloroform	(1:1)	1000 cm^3
(iv) Chloroform	Pure	1500 cm^3
(v) Chloroform/Methanol	(95:5)	2000 cm^3
(vi) Chloroform/Methanol	(9:1)	1000 cm^3
(vii) Methanol	Pure	200 cm^3

Eluents were examined on thin layer plates and combined into three major fractions. Fraction (A) was brought out by petroleum ether, fraction (B) by chloroform: methanol (9:1) and fraction (C) by chloroform: methanol (95:5). These fractions from the column were subjected to preparative thin layer chromatography for further separation into pure compounds. Fraction (A) yielded compound CH1, fraction (B) yielded compound CH2, while fraction (C) yielded both compounds CH3 and CH4.

Alkaloid Extract (III)

1. Concentrated
2. Column (alumina)

Pet Ether (40-60°C)   CHCl₃: MeOH (9:1)   CHCl₃: MeOH (95:5)

Fraction A   Fraction B   Fraction C

1. Concentrated
2. Preparative TLC

1. Concentrated
2. Preparative TLC

CH1   CH2   CH3   CH4

Oily white liquid   Violet solid   Pale yellow solid   Bright yellow solid
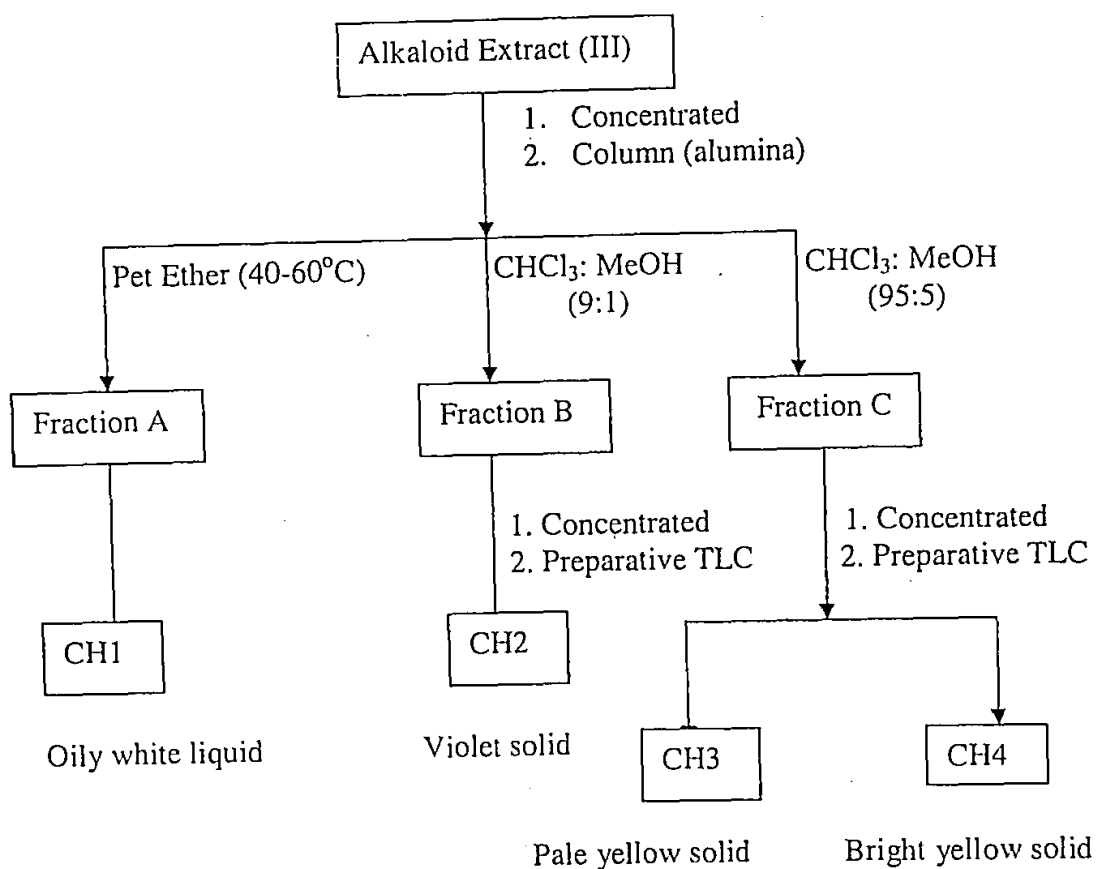
Fig. 20: Flowchart of the Isolation of Alkaloid Extract

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Preparative Thin Layer Chromatography (Bobbit *et al.*, 1964; Stock *et al.*,)

The glass plates, 20 x 40 cm, were washed with chromic acid, detergent, and then water and dried in the oven. The dried plates were assembled on their support and finally wiped with cotton wool moistened with acetone to remove all grease.

Sixty grams of silica gel G (type 60 Merck) were weighed into a beaker. One hundred and twenty cubic centimetres of water was added and the mixture stirred vigorously for about a minute. The slurry was quickly put into a spreader placed at one end of the plates and then spread over the plate in the usual way. The plate was then left to dry at room temperature. The thickness of a layer prepared was one millimetre. When required for use, the plates were baked in the oven at 120°C for at least one hour.

The fractions obtained by eluting the column were found to contain more than one component. These mixed fractions were purified using preparative TLC. Silica gel was used as the adsorbent. The mixture was dissolved in appropriate solvent and applied in a thin band at about two centimetres from one edge of the plate using Desaga apparatus. The bands were located under ultraviolet lamp. The compound (CH1) was a white oily liquid and gave one spot on a chromatogram. An attempt at separating fractions from chloroform: methanol (9:1) yielded a violet compound (CH2) and that of chloroform: methanol (95:5) yielded both (CH3) a pale yellow crystal and bright yellow crystal (CH4).

Isolation of CH1

Thirty bottles of the petroleum ether fractions were collected and out of these 12 showed a single spot on the chromatogram and so they were combined and concentrated. This was labelled as fraction A. These fractions yielded a white oily liquid sample CH1 (0.02g) and an Rf of 0.2. To ascertain the purity it was taken through a series of thin layer chromatographs.

Isolation of CH2

Ten bottles of the chloroform: methanol (9:1) fraction were collected and concentrated to form fraction B. This fraction was dissolved in chloroform and separated on preparative plates. The mobile phase was chloroform: methanol (9:1). The main band Rf 0.3 violet in colour was scraped with spatula and eluted with methanol. The methanol solution was concentrated and examined on thin layer plates. This afforded the single component CH2 (0.003g) with a melting point of 165-168°C.

Isolation of CH3

A total of 20 bottles of fractions were collected for the solvent system chloroform: methanol (95:5) and concentrated. This was labelled as fraction C. It was dissolved in chloroform and separated on preparative plates. The mobile phase was chloroform: methanol (9:1). The first band Rf 0.4 was scraped with spatula and eluted with methanol. The methanol solution was concentrated and

examined on thin layer plates. This afforded a pale yellow coloured compound CH3 (0.002g).

Isolation of CH4

The main band from the chloroform: methanol (fraction C) with Rf 0.6 was also scraped with a spatula and eluted with methanol. The methanol solution was concentrated and examined on thin layer chromatographic plates. This afforded the bright yellow compound CH4 (0.45g) with a melting point of 248-251°C.

Table 6: Isolated components and their Rf values Solvent system CHCl₃: MeOH

Components	Ratio	Rf x 100
CH2	9:1	300
CH3	95:5	400
CH4	95:5	600

Melting point Determination

The melting points of all the compounds were taken using the electrothermal melting point instrument (9100): The values obtained for the various components are tabulated below (Table 7).

Table 7: Melting points of components isolated

Components	Melting point (^{o}C)
CH2	165-168
CH4	248-251

Column Chromatography (CC)

This was run using an alumina (for CC) with various solvent systems of different polarities and ratios.

Thin Layer Chromatography (TLC)

This was performed on silica gel (for TLC) with plate thickness of 0.25 mm and 10 cm running distance. The components were visualized under UV light and in iodine vapour.

Bioassay on the Extract CH4

As a means of sterilization, a Bunsen burner was lighted and allowed to burn for five minutes to create a clean sterilized working environment. With a well sterilized microbiological loop, colonies from pure culture isolation plates were taken into peptone water (water-soluble protein derivatives). The inoculated peptone water was poured on the sensitivity agar: *Escherichia coli* and *Pseudomonas aeruginosas* and the excess discarded. Four different susceptibility discs (5mm in diameter) were impregnated with 10, 5, 2.5 and 1.0μg of the isolated quindoline compound dissolved in MeOH (10 cm^{3}) and placed on the

sensitivity agar. This was then incubated in the incubator at a temperature of 37°C. The growth pattern of the microorganisms was observed for 18hours.The control experiment was done with methanol (10 cm^3) without the quindoline compound.

CHAPTER FOUR

RESULT AND DISCUSSION

Preliminary Tests on Crude Extract

The table below summarizes the results on the Preliminary investigations done on the crude extract.

Table 8: Preliminary Tests on Crude Extract

Test	Observation	Inference
1. Alkaloids Test		
(a) Dragendorff's reagent added in drops on a filter paper impregnated with sample dried and washed with water.	Persistent appearance of orange colour formation	Alkaloids present
(b) Mayer's reagent added to sample.	Presence of precipitate	Alkaloids present
(c)Wagner's reagent added to sample.	Presence of white Precipitate	Alkaloids present

Table 8 *continued*

Test	Observation	Inference
2. Anthraquinone Test		
0.3g of 80% ethanol extract evaporated to dryness. The filtrate and 5cm^3 of benzene and 2.5 cm^3 of ammonia test.	There was no red colour in the alkaline layer.	Absence of anthraquinone
3. Flavonoid Test		
4 cm^3 of ethanol sample heated and Magnesium turnings added alongside with 0.5 cm^3 of Conc. HCl.	No red or orange colour was formed.	Flavonoids absent

Result of Column Chromatography

Table 9: Nature of isolates from column.

Solvent System	Ratio	Result	Label	RF
Pet-ether (40-60°C)	100%	Oil	CH1	0.2
Chloroform: methanol	9:1	Violet	CH2	0.3
Chloroform: methanol	95:5	Pale yellow	CH3	0.4
Chloroform: methanol	95:5	Bright yellow	CH4	0.6

Thin layer Chromatography check on isolated sample

This was used to select the best solvent system for the isolation of major components. It was used to determine from the beginning the number of major components present in the sample. Fig. 18 gives the summary of the test.
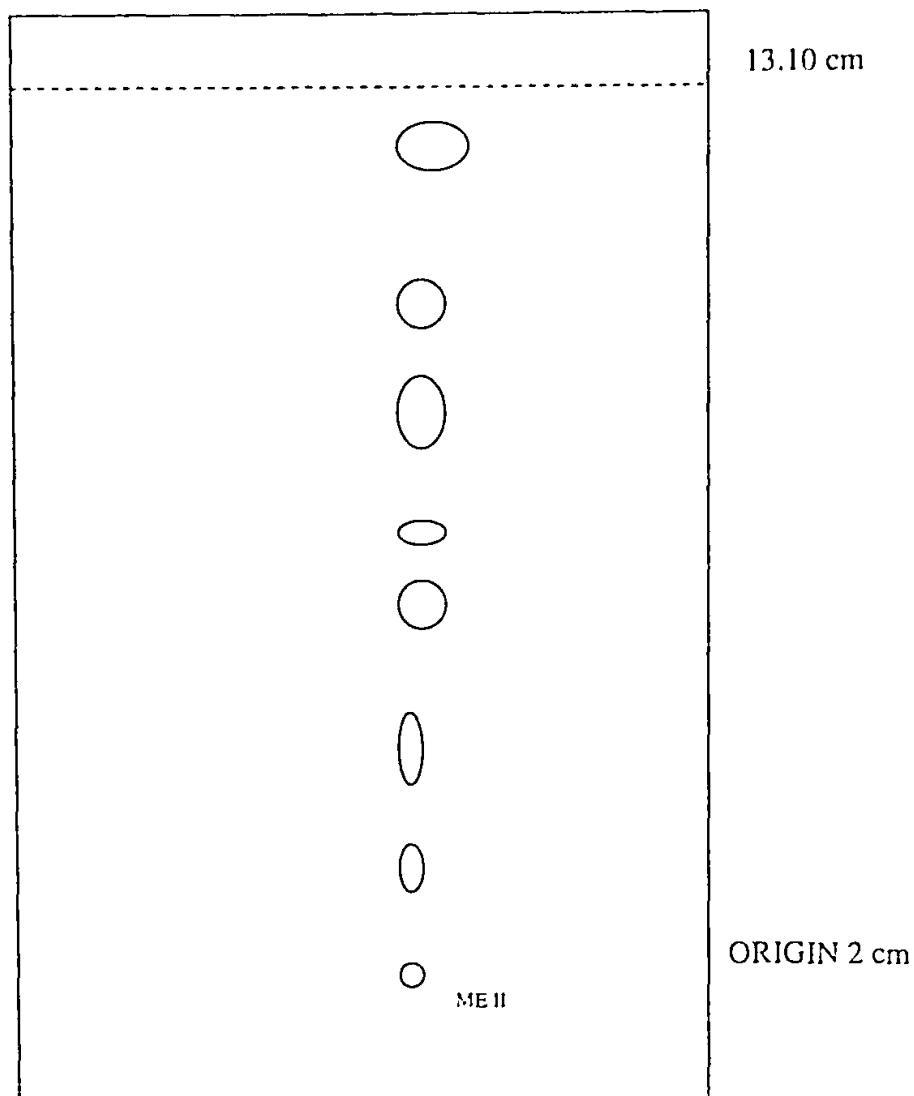

Fig. 18: Thin Layer Chromatogram of Methanol Extract (II) on Silica Gel. Solvent: -Chloroform: Methanol (95:5)

Chloroform: methanol (95:5) solvent system gave good separation with 7 components that were well separated from each other. However, a kind of gradient elution using chloroform: methanol ratio was employed to elute the sample.

Purity Examination of the Isolated Compounds

A thin layer chromatography check was performed on all the isolated compounds to ascertain their purity. **Fig. 19** shows chromatogram for the isolated components.

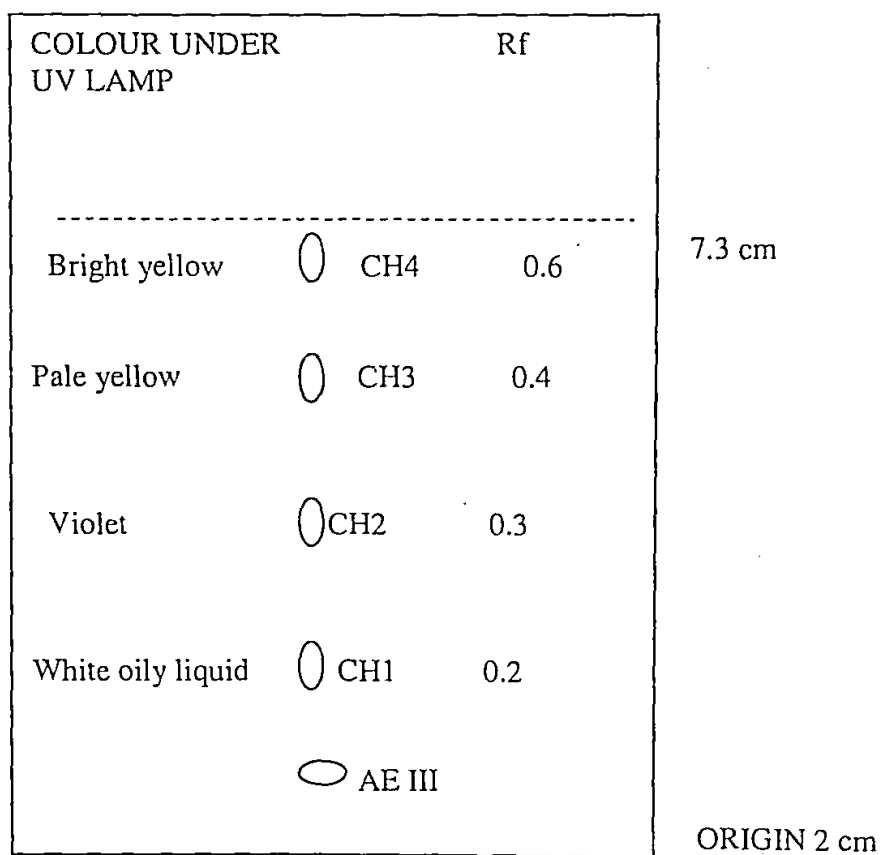| COLOUR UNDER UV LAMP		Rf	
- -			
Bright yellow	◯ CH4	0.6	7.3 cm
Pale yellow	◯ CH3	0.4	
Violet	◯CH2	0.3	
White oily liquid	◯ CH1	0.2	
	◯ AE III		
			ORIGIN 2 cm

Fig. 19: Thin Layer Chromatogram of Isolated compounds (IV) on Silica Gel. Solvent: - Chloroform

Identification of the Isolated Compounds

The isolates CH1, CH2, CH3 and CH4 were subjected to phytochemical screening methods proposed by Farnsworth *et al* (1977) to confirm the presence of alkaloids. The following alkaloid tests (Wagner's, Mayer's and Dragendorff's) were performed on the isolated compounds. All the samples were positive to alkaloids tests. Apart from sample CH1 that was liquid and oily, all the samples were solids. In the case of the Dragendorff's reagent, there was a persistence appearance of an orange colour, which indicated the presence of alkaloids in the isolated compounds. That of Mayer's and Wagner's showed a yellow-white precipitate, an indication that alkaloids were present in these compounds.

Spectral Analysis

The four samples (CH1, CH2, CH3 and CH4) were all sent outside for the spectral analysis to be performed on them. Spectral values were however, obtained for only samples CH2 and CH4 and so no further work were done on both samples CH1 and CH3. This section therefore deals with the spectral results received on samples CH2 and CH4.

Ultraviolet (U.V) Spectroscopic Analysis

The ultraviolet spectral properties of sample CH4 were determined as $\upsilon\lambda_{max}$ (methanol) nm 272, 339, 399.

Mass Spectrometric Analysis

Mass spectrometric analysis was performed on sample CH4. The mass spectrum of CH4 (Appendix IV) shows the base and molecular mass peak of 218. The other prominent peaks in the mass spectrum of CH4 are m/z 89, 90, 109 and 190.

Nuclear Magnetic Resonance (NMR) spectroscopy

Proton-magnetic resonance (^{1}H-NMR) and Carbon-13 Nuclear magnetic resonance (^{13}C-NMR) were performed on the two samples (CH2 and CH4). The peaks of ^{1}H-NMR and ^{13}C-NMR are quoted in ppm.

This includes 1D Attached Proton Test (APT), which was used to identify the number of Hydrogen atoms attached to each carbon atom in a ^{13}C spectrum. In addition, 2-Dimensional spectroscopy such as ^{1}H-^{1}H HMBC was performed on the sample labelled, CH2 (Appendix IXa-IXg). The ^{1}H-NMR spectrum of (CH4) reveals nine protons in different chemical environments. The resonance (δ) and their corresponding protons with their multiplicities are tabulated in the tables

Table 10: Chemical shifts (δ) and coupling constants data
^{1}H-NMR for compound CH4 in CD$_3$OD

Position of proton	Resonance (δ) ppm
8	7.70q
4	8.35d
1	8.2d
3	7.90t
2	7.60d
6	8.80s
7	7.35t
11	8.50d
9	7.70q
NH	-

Table 11: ^{13}C-NMR chemical shift assignments (δ) for compound CH4 in CD$_3$OD

Position of Carbon	Resonance (δ) ppm
5a	146.9
9a	138.6
4a	136.9
10a	134.8
8	134.5
4	131.9
1	129.6
11a	127.9
3	127.4
2	124.4
6	123.9
5b	122.2
7	122.0
11	115.8
9	113.6

Table 12: ^{13}C-NMR chemical shift assignments (δ) for compound CH2 in CD$_3$OD

Position	Chemical shift (δ) ppm
5a	146.428
9a	138.428
4a	135.773
10a	133.956
8	133.642
4	132.520
1	129.650
11a	126.993
3	126.579
2	125.372
6	124.663
5b	121.554
7	116.837
11	113.916
9	112.829

Table 13: Chemical shifts (δ) and coupling constants data
^{1}H-NMR for compound CH2 in CD_3OD

Position	Chemical shift (δ) ppm
2	7.58
3	7.86
4	7.94
5	8.20
6	8.48
7	8.65
8	8.78
9	9.14

Table 14: ^{1}H- ^{1}H correlation (COSY) data on Compound CH2 in CD_3OD

H	H
2	4,8
4	5,6
5	7
3	4

Discussion

The preliminary test performed on the plant suggested the presence of alkaloids. This actually confirmed what has been reported in literature. Column chromatography on the alkaloid extract yielded four components CH1, CH2, CH3 and CH4. CH1 was liquid and oily. CH2, CH3 and CH4 were solids. CH2 (violet, m.pt 165-168°C) was isolated using chloroform: methanol 9:1 ratio. CH3 (pale yellow) was isolated from the chloroform: methanol 95:5 ratio after preparative TLC. The last isolate labelled CH4 (bright yellow, m.pt 248-251°C) was also isolated from the chloroform: methanol 95:5 ratio after preparative TLC.

The structures of the compounds were deduced mainly from the experimental spectral data and comparing it with those proposed by Spitzer *et al.*, (1991).

Identification of CH4

CH4 was isolated as a bright yellow amorphous substance of melting point 248-251°C. It gave a positive test result for alkaloids with Dragendorff's, Wagner and Mayer's reagents.

The mass spectrum of CH4 (Appendix IV) gave a molecular ion peak at 218, which is in agreement with molecular formula of $C_{15}H_{10}N_2$. The fragmentation pattern (**Fig. 19**), according to the peaks that appear in the spectrum goes to confirm the structure of CH4. The 15 carbon atoms were depicted in the ^{13}C-NMR spectrum and distributed into 15 aromatic carbon atoms 9 CH and 6 quaternary sp^2 carbons. The 15 aromatic carbons absorbed between the ranges

146.9 - 113.6 ppm. The 6 quaternary sp^2 carbons are located at these positions: 4a, 5a, 10a, 11a, 5b and 9a [1.12]. The ^{13}C-NMR shows 15 peaks, which indicates the absence of any symmetry (table 11). The evidence from the NMR spectra was very instrumental in assigning the structure of CH4. The structure was confirmed by comparing the ^{1}H-NMR and ^{13}C-NMR spectra with that provided by Spitzer *et al.* (1991) and also with quindoline from ACD/Labs/Chem./Sketch (a chemistry software for drawing and elucidating Organic structures). The ^{1}H-NMR and the ^{13}C-NMR chemical shifts reported here (table 10 and 11) are in agreement with what was reported by Spitzer *et al.*, 1991. Our data from the NMR studies confirmed the structure proposed originally for quindoline [1.12].
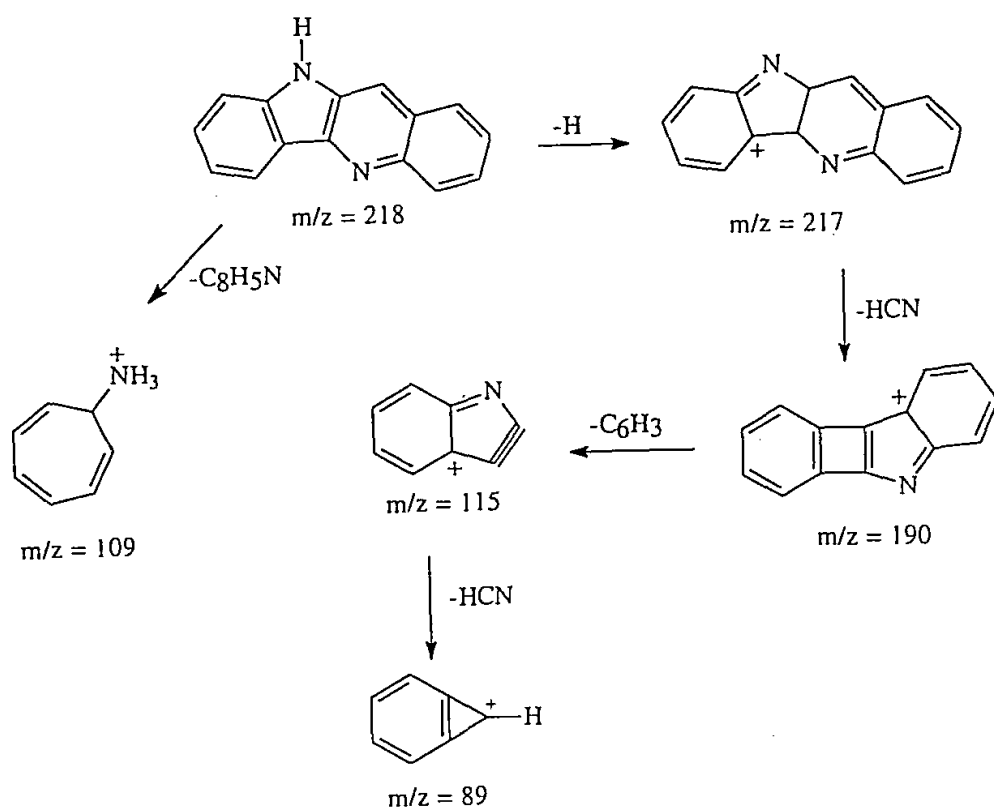
Fig.16: Fragmentation pattern of the compound quindoline

Identification of CH2

CH2 was isolated as a violet substance of melting point 165-168°C. It gave a positive test result for alkaloids with Dragendorff's, Wagner and Mayer's reagents. The structure of the compound was determined by comparing it with values from literature as reported by Spitzer *et al.* (1991).

We attempted to apply various spectra techniques to obtain the total assignment of the proton and carbon nmr spectra of the alkaloid cryptolepine [1.11] (5-methyl-5H-indolo[3,2-b]quinoline), which is derived from *Cryptolepis sanguinolenta* an endemic medicinal for the treatment of malaria in West Africa.

Within the proton spectrum, the only resonance assignable by inspection is the H11 singlet resonating furthest downfield at 9.14 ppm (Table 11). It was also obvious that the balance of the resonances in the aromatic region of the spectrum comprised two four-spin systems, which is consistent with the structure of [1.11]. The COSY spectrum (Appendix VIIa-VIIb) allowed the sub-grouping and establishment of vicinal neighbour relationships within the two four-spin systems.

The most important assignment information was derived from the HMBC (Heteronuclear multiple bond correlation). The HMBC establishes the direct or one-bond correlation. It provides the means of unequivocally assigning all of the proton and carbon resonances of the spectra by orienting the individual spin systems relative to one another through common couplings to identifiable points in the molecular structure. The most convenient starting point for the assignment is the H11 singlet. As observed in the HMBC spectrum presented in (Appendix

IXa-IXg), connectivities are observed from H11 to the carbons resonating at 146.43, 135.77 and 129.65 ppm.

The carbon resonating at 146.43 ppm showed no other connectivities and consequently may be tentatively assigned as C5a. The carbon resonating at 135.77 ppm exhibited connectivities to the "doublet" resonating at 8.49 ppm and the multiplet resonating at 8.18 ppm. From the COSY spectrum (Appendix VIIa-VIIb) it can be concluded that the protons at 8.49 and 8.18 ppm are meta to one another. Given this information, the only carbon that can be uniquely coupled to the three protons in question is C4a, as shown by the arrows used to denote these connectivity pathways on [1.11a].
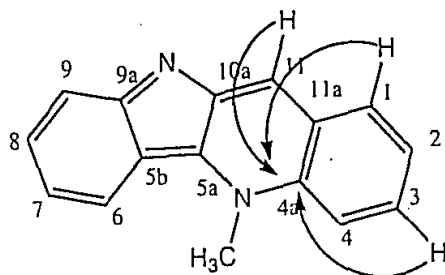
[1.11a]

Using the connectivities just established as a point of departure, the balance of the protonated carbons, C1 through C4, may be unequivocally assigned as may be their corresponding, directly bound protons. It is worth noting that H1 exhibited a long-range correlation to the C11 resonance and, conversely, H11 was long-range coupled to C1. In the absence of a common quaternary coupling partner, the two-spin systems in question could be oriented to one another from this set of coupling pathways. Looking at the long-range correlations of H11, it can be said that the only correlation to be considered is the carbon resonating at

146.43 ppm. The coupling possibilities that remain are $^2J_{CH}$ couplings to C10a or C11a and $^3J_{CH}$ to C5a. Of these, the coupling to C11a may be eliminated from consideration because C11a is assignable as the carbon resonating at 126.99 ppm on the basis of long-range couplings to H2 and H4. Of the two remaining coupling pathways, because $^3J_{CH}$ couplings are generally much larger than the $^2J_{CH}$ counterparts, it is logical, to assign the resonance at 146.43 ppm as C5a. Although tentatively assigned as C5a, the assignment of the quaternary carbon can be unequivocally made on the basis of long-range couplings to the N-methyl group. While the N-methyl proton resonance was not included in the HMBC spectrum this information was acquired from a report by Tackie *et al.* (1991).

Assignments made thus far account for three of the six quaternary carbon resonances, leaving those at 138.43, 133.96 and 121.55 ppm to be assigned. Returning to the premise that H7, *para* to the "indole" annular nitrogen, would resonate furthest upfield at 5.12 ppm based on comparison to normal indole proton chemical shifts, we note that this proton was also long-range coupled (Appendix VIIb) to the quaternary carbon resonating at 121.55 ppm, as was the protons resonating at 7.85 ppm. The two protons now in question were meta to one another on the basis of the COSY spectrum (Appendix VIIa-VIIb). These correlations lead to an assignment of the carbon resonating at 121.55 ppm as C5b, the chemical shift of which is also quite reasonable for its location in the structure of the molecule. This confirms the assignment of H7 at 5.12 ppm and the assignment of H9 at 7.85 ppm.

Of the two remaining quaternary carbons, the carbon resonating at 138.43 ppm was observed to long-range couple (Appendix VIIb) to the protons resonating at 8.76 and 7.85 ppm. Given the relationship of these protons to H7 and H9, they are assigned as H6 and H8, respectively, thereby assigning the quaternary carbon as C9a. These long-range coupling pathways are shown by [1.11 b]
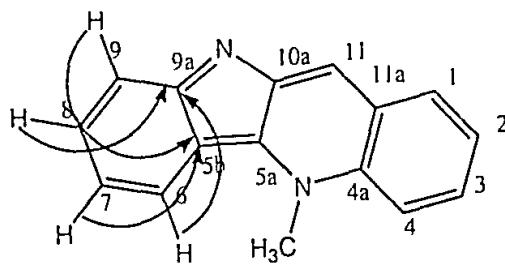
[1.11b]

The sole remaining quaternary carbon resonating at 133.95 ppm exhibited no long-range couplings in the HMBC spectrum and is thus assigned as C10a. The lack of correlation responses for this carbon is not particularly surprising: the only coupling reasonably available to it is to H11 *via* $^2J_{CH}$ (Tackie *et al.*, 1991).

Bioassay of Quindoline

The result showed that the higher concentrations of the isolated sample (CH4) inhibited the growth of *Escherichia coli*. However, *Pseudomonas aeruginosas* was resistance to all the concentrations of the isolated sample (CH4). In all cases for the control containing only MeOH, no zones of inhibition were observed.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

Conclusion

The alkaloids CH2 and CH4 were isolated from the stem of the plant. Characterization of the compounds (CH2 and CH4) was made possible due to the application of advanced modern spectroscopy methods. The structures of the compounds were elucidated by comparing the experimental spectral data with those described by Spitzer *et al.* (1991). Although the isolated compounds are not new, these compounds have been mainly isolated from the roots of the plant. In this experiment, however, they were isolated from the stem (bark and wood) extract of the plant.

Though structure elucidation could not be achieved for samples CH1 and CH3 in this work, it is suggested here that further work should be done to ascertain the authentic structures of these compounds. Apart from the normal Chromatographic methods that have been employed in isolating CH1 and CH3, it is suggested here that the oil sample (CH1) be subjected to both Gas chromatographic (GC-MS) and High performance liquid chromatographic (HPLC) analysis in order to characterise it.

From the many compounds known to be isolated from the plant, it can, properly, be concluded that the many claims as to the uses of the plant cannot be

disputed. Most of the claims have been attributed to some compounds of the plant. The plant is synonymous with the treatment of malaria.

Recommendation

It is recommended that further work on the plant should be directed towards the oils in the stem of the plant. Therefore, it is suggested that further thorough and scientific pharmacological studies should be made on the isolated oils from the plant in order to find out if it has any biological importance.

Analytical instruments should therefore be provided at the Chemistry Department of the University of Cape Coast to ease research work into natural products of this nature. Although this appeal may sound to some people as old fashion, it is my hope and desire that this perennial lack of equipment will be resolved once and for all. These instruments should be mainly nuclear magnetic resonance spectrometer, mass spectrometer and ultraviolet spectrometer. This will encourage most graduate students to complete their research work on time and hence make the department appealing to students who want to do their masters in chemistry, more especially Organic Chemistry.

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APPENDICES

APPENDIX I: PHYTOCHEMICAL METHODS

Phytochemical Screening Methods

Ultimately, the goal in surveying plants for biologically active or medically useful compounds is to isolate one or more constituents responsible for a particular activity.

There have been two main approaches by investigators primarily interested in the discovery of new biologically active plant principles, namely, phytopharmacological and phytochemical approaches.

Phytopharmacological approaches consider those methods, which involve, as a first step, the observation or detection of biological activity induced by plant products.

Malone and Robichaud (1962) have remarked on general screening as follows: "the basic premise of pharmacological screening is not to allow true biological activity to go undetected even though the activity maybe new, unexpected and unique. The initial screening procedure must unequivocally establish this phytopharmacological activity as well as the phytochemical nature of the constituents, in order to indicate the course of further and more specific pharmacological evaluation. "These workers proposed a "Hippocratic screen", utilizing normal anaesthetized rats for the detection of biological activity in crude plant extracts.

Phytochemical screening describes methods used for the detection of phytochemical classes of compounds, examples of which are known to elicit some

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desirable biological response. Thus, some investigators will select initially only alkaloid containing plants for study on the premise that (a) alkaloids normally exert some type of pharmacological activity on the central nervous system and (b) the greatest majority of natural products used in medicine today are alkaloid in nature. Along similar lines, an investigator may decide to pursue a phytochemical approach leading to isolation of phytoconstituents: saponins, flavonoids, tannins, coumarins or anthraquinones.

Methods of Extraction and Isolation

Ideally, fresh plant tissues are used for phytochemical analysis and are plunged into boiling alcohol within minutes of its collection. This treatment 'kill' the plant tissue, i.e. prevent enzymic oxidation or hydrolysis occurring. When plant material is to be transported to place of use, it is stored in plastic bag, which remain in good condition for analysis several days after collection. More commonly, plants are dried before extraction. In drying, it is essential that the drying operation is carried out under controlled condition s to avoid too many chemical changes occurring. The plant material should be dried as quickly as possible, without using high temperatures (40°C).

The classical chemical procedure for obtaining organic constituents from dried plant tissue (heartwood, dried seeds, root, leaf etc.) is to continuously extract powdered material in a soxhlet apparatus with a range of solvents, staring in turn with petroleum ether, ether and chloroform (to separate lipids and terpeniods) and then using alcohol and ethylacetate for more polar compounds.

The extract obtained is concentrated in vacuo. This is usually carried out in a rotary evaporator, which will concentrate bulky solutions down to small volumes at temperatures between 30 and 40°C. The concentrated extract may deposit crystals on standing. If so, these are collected by filtration and their homogeneity tested by chromatography.

Methods of separation

The separation and purification of plant constituents are mainly carried out using one or other, or a combination of three chromatographic techniques: paper chromatography (PC), thin layer chromatography (TLC) and gas liquid chromatography (GLC). The choice of technique depends largely on the solubility properties and volatilities of the compounds to be separated. It is often difficult to follow general or expected solubility rules for a given class of phytoconstituents since there are often substances of unknown character present in crude plant extracts that affect solubility. For example, n-hexane soluble extractives from *Catharanthus lanceus* (Farnsworth, 1966) were found to be rich in alkaloids. Subsequent isolation of individual alkaloids from the crude mixture proved them to be totally insoluble in n-hexane. The alkaloids were found to occur in the plant dissolved in lipid material, the latter being soluble in n-hexane. It is therefore necessary that extract residues be examined with a variety of solvents to determine whether abnormal solubility phenomena have occurred.

Paper chromatography is particularly applicable to water soluble plant constituents, namely the carbohydrates, amino acids, nucleic acid bases, organic acids and phenolic compounds (Harborne, 1973).

Thin layer chromatography is the method of choice for separating the lipids, steroids, carotenoids, quinines and chlorophylls (Harborne, 1973).

Gas liquid chromatography finds its main application with volatile compounds, fatty acids, mono and sesquiterpenes and hydrocarbons (Harborne, 1973).

The volatility of higher boiling plant constituents can, however, be enhanced by converting them to esters and/ or trimethylsilyl ethers so that there are few classes, which are completely unsuitable for GLC separation.

There is also a considerable overlap in the use of the above techniques and often a combination of PC and TLC or TLC and GLC may be the best approach for separating a particular class of plant compound. The GLC apparatus can be set up in such a way that the separated components are further subjected to spectral or other analysis. Most frequently, GLC is automatically linked to mass spectrometry (MS) and the combined GC-MS apparatus has emerged in recent years as one of the most important of all the techniques for phytochemical analysis.

Methods of Identification

In identifying a plant constituent once it has been isolated and purified, it is necessary first to determine the class of compound and then find out which

particular substance it is within that class. Its homogeneity is checked beforehand, i.e. it should travel as a single spot in several TLC and /or PC systems. The class of compound is usually clear from its response to colour tests, its solubility and Rf properties and its ultraviolet spectral characteristics.

Complete identification within the class depends on measuring other properties and then comparing these data with those in the literature. These properties include melting point (for solids), boiling point (for liquid), optical rotation for optically active compounds and Rf.

The most informative data on a plant substance are its spectral characteristics; these include ultraviolet (UV), infrared (IR), nuclear magnetic resonance (NMR) and mass spectral (MS) measurements. If authentic material is available, direct comparison is carried out as final confirmation. If not available, careful comparison with literature data may suffice for its identification. If a new compound is present, all the above data should be sufficient to characterise it. With new compounds, however, it is preferable to confirm the identification through chemical degradation or by preparing the compound by laboratory synthesis.

Alkaloid Screening

The alkaloids, of which some 5,500 are known, comprise the largest single class of secondary plant substances.

Alkaloids are more or less toxic substances, which act primarily on the central nervous system, have a basic character, contain heterocyclic nitrogen, and

are synthesized in plants from amino acids or their immediate derivatives. In most cases they are limited distribution in the plant kingdom. They are usually colourless, often optically active substances and most are crystalline.

The fact that most alkaloids are basic and therefore soluble in aqueous acids suffices to separate them from the host of water insoluble products found in plants. Thus most workers believe that extraction with acidulated water can result in crude extract, which can be tested directly with one or more standard alkaloid precipitating reagents (Farnsworth, 1966). Other workers feel that the presence in such an extract of materials that are capable of giving false-positive alkaloid tests necessitate a purification procedure before valid results can be obtained (Farnsworth, 1966). This is usually accomplished by the addition of base and subsequent extraction with a water-immiscible organic solvent. The choice of organic solvent in practice is limited to ether or chloroform. The organic extract can be tested by application to filter paper, drying, and dipping or spraying with an alkaloid detecting reagent that gives a chromogenic response with alkaloids. If the latter method is not preferred, the organic solution can be re-extracted with dilute acid and alkaloid precipitating reagents added to separate portions of this acid extract (Farnsworth, 1966).

The aqueous acid solution is rendered alkaline with ammonia at 60° or sodium carbonate. The basification with ammonia at 60° is advocated because some alkaloids are not precipitated at room temperature (sodium hydroxide is used when phenolic alkaloids are known to be absent). The basic constituents are recovered by extraction with ether or chloroform. The aqueous alkaline mother

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liquor is made acidic (pH 4) with 10% hydrochloric acid and the quaternary base can be precipitated with ammonium reineckate after the procedure of (Hogg, 1961) for further characterization.

Alkaloid Detecting Reagents

For detecting alkaloids in phytochemical screening, two types of reagents are used: alkaloid precipitants and spray or dip reagents.

(a) Dragendorff's Reagent

0.85g of basic bismuth nitrate dissolved in 40cm^3 water and 10cm^{3}glacial acetic acid followed by addition of 8g of potassium iodide dissolved in 20 cm^3 of water.

    a. Solution A: - 0.3g bismuth sub nitrate plus 1 cm^3 of 25%HCl and 5 cm^3 water.

    b. Solution B: -3g potassium iodide in 5 cm^3 water. 5 cm^3 A + 5 cm^3 B + 5 cm^3 HCl + 100 cm^3 water. This is for spraying

(b) Mayer's Reagent

Mercuric chloride (1.358g) is dissolved in water (60 cm^3). To this added, potassium iodide (5.0g) dissolved in water (10 cm^3). The mixture is diluted to 100 cm^3 with water.

(c) Wagner's Reagent

Potassium iodide (2.0g) added to (1.27g) of Iodine and then dissolved in distilled water to make 1000 cm^3 of solution.

(d) Ammonia T.S.

Ammonium hydroxide (28%) – 35.7 cm^3.

Distilled water – 100.0 cm^3.

(e) Borntrager Test

The powdered material is macerated with an immiscible organic solvent and after filtration aqueous ammonia or caustic soda is added. A pink, red or violet colour in the aqueous layer after shaking indicates the presence of anthraquinones.

(f) Schinoda's Test

To the alcoholic extract is added magnesium tunings followed by concentrated HCl. Absence of pink colouration indicates the absence of flavonoids.

(g) Preparation of TLC Plates

Appropriate amount of the adsorbent (silica) was weighed and the required volume of water was added. The mixture was triturated gently in one direction to a smooth paste. The paste was poured into a spreader and

run over the glass plates arranged horizontally and held in place by a metal frame. The thickness of the adsorbent on the plates is determined by allowing the sides with the right thickness facing away from the direction of the spreading such that, the side runs over the adsorbent. The plates are then air dried for about 30 minutes and then packed in metal rack and activated in the oven at 110°C for 1-2 hours.
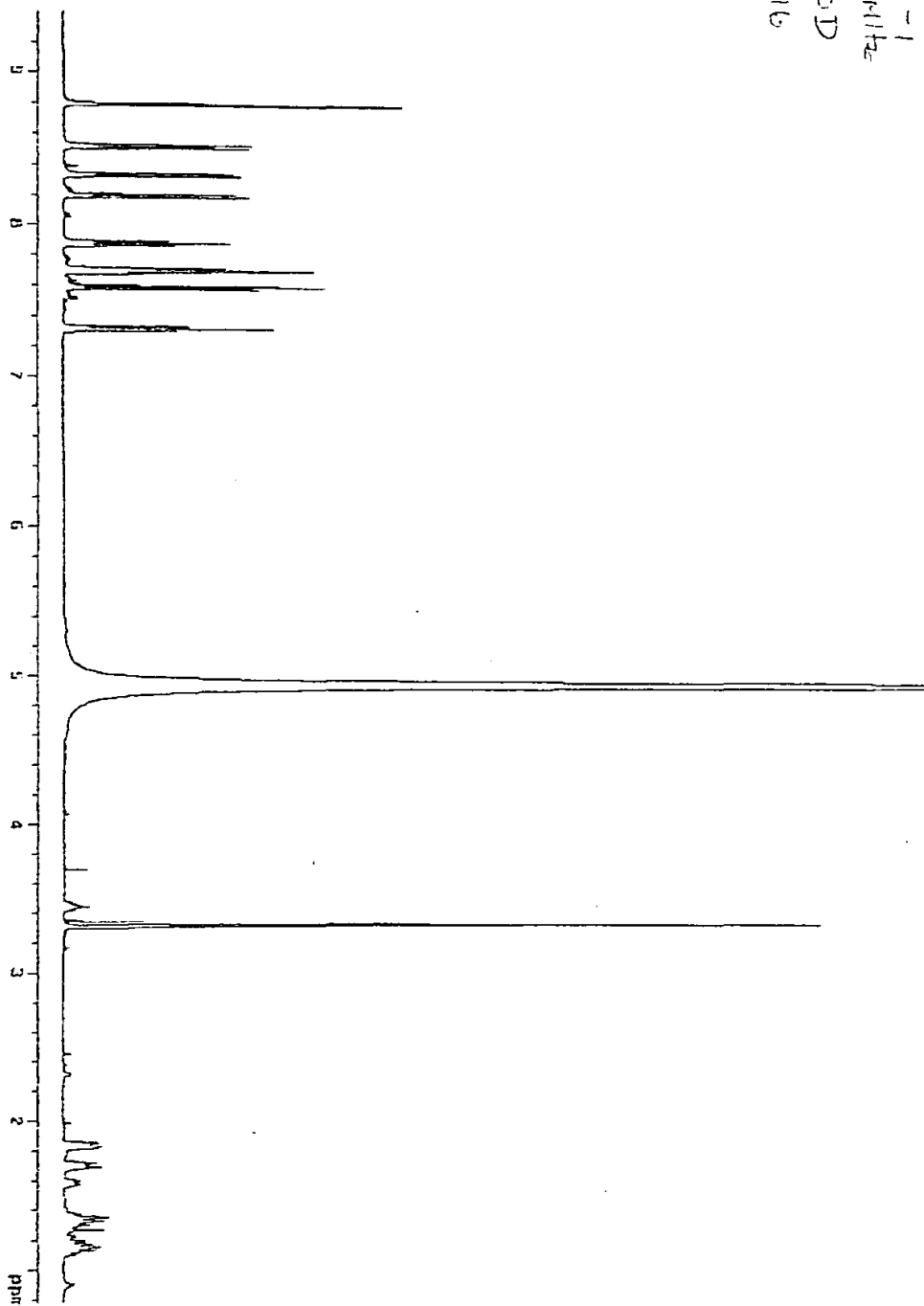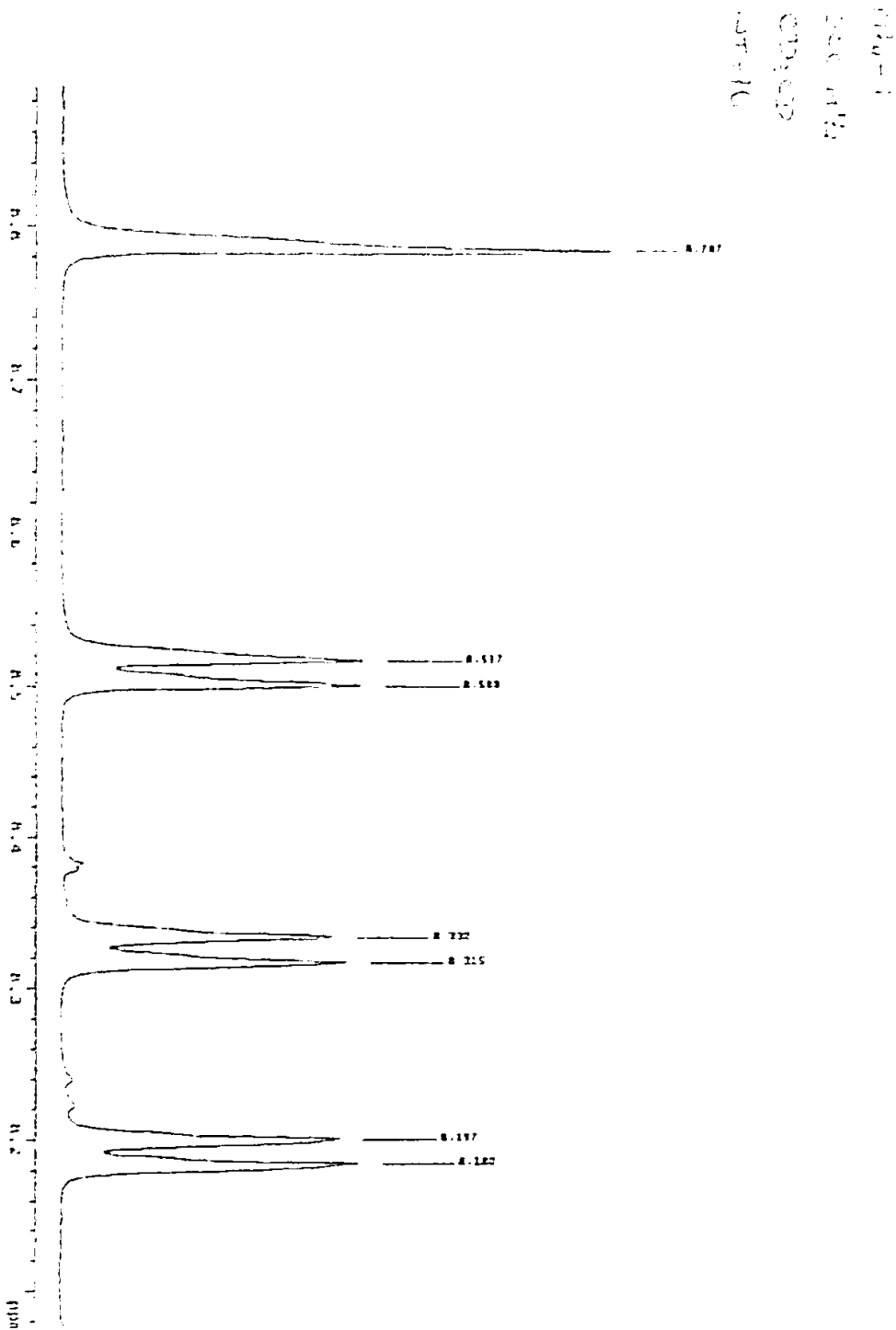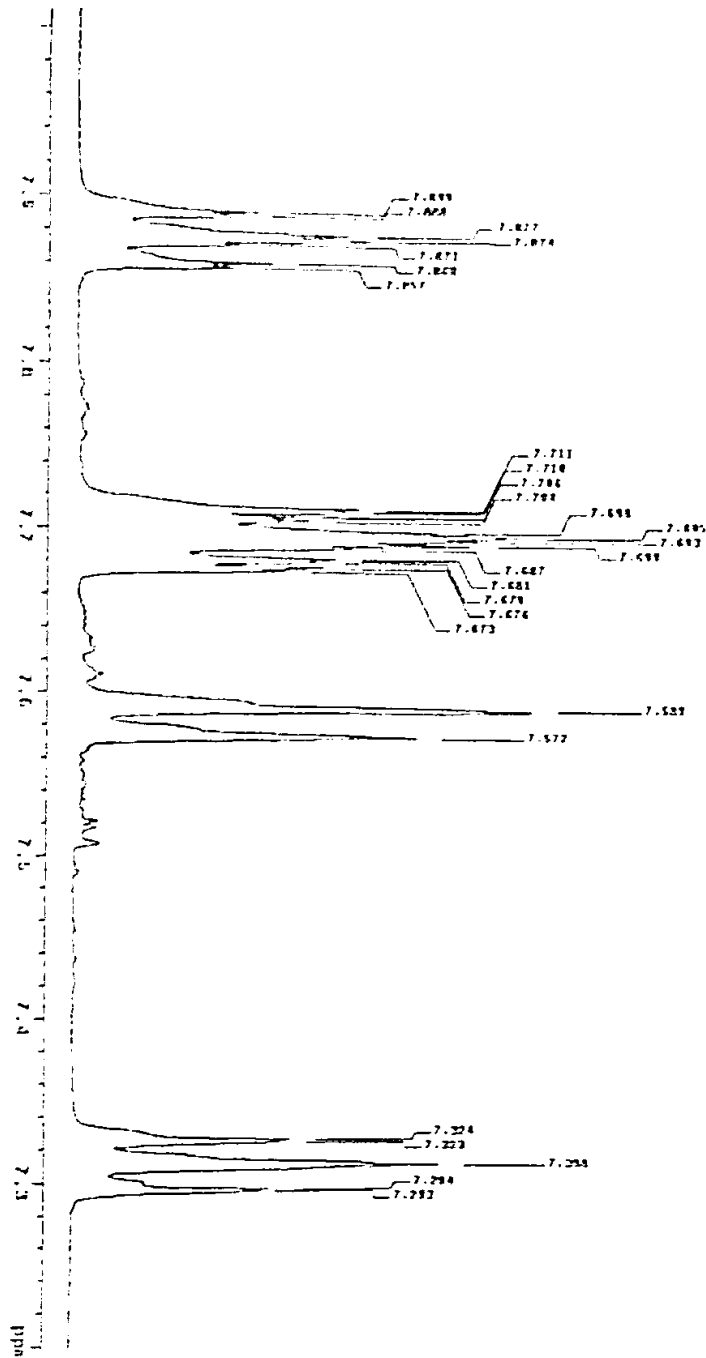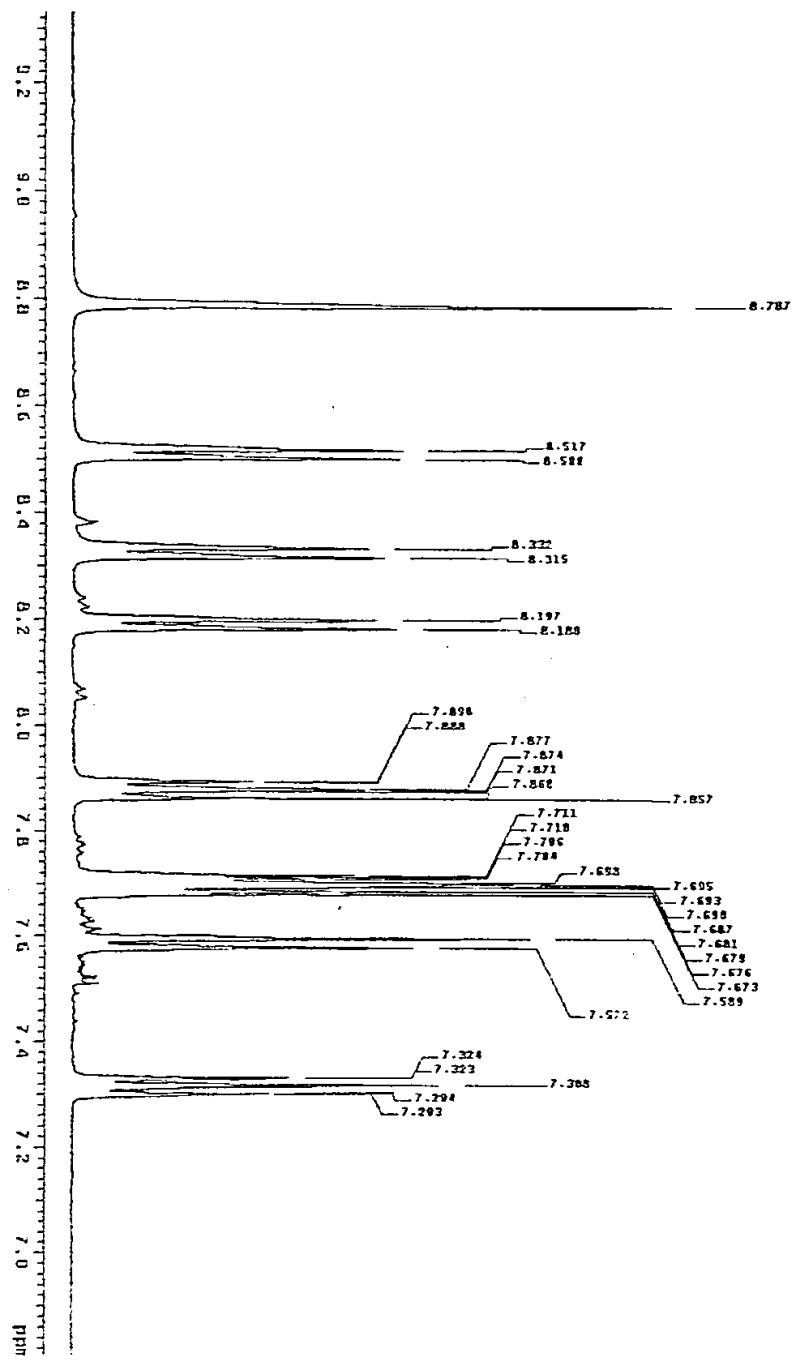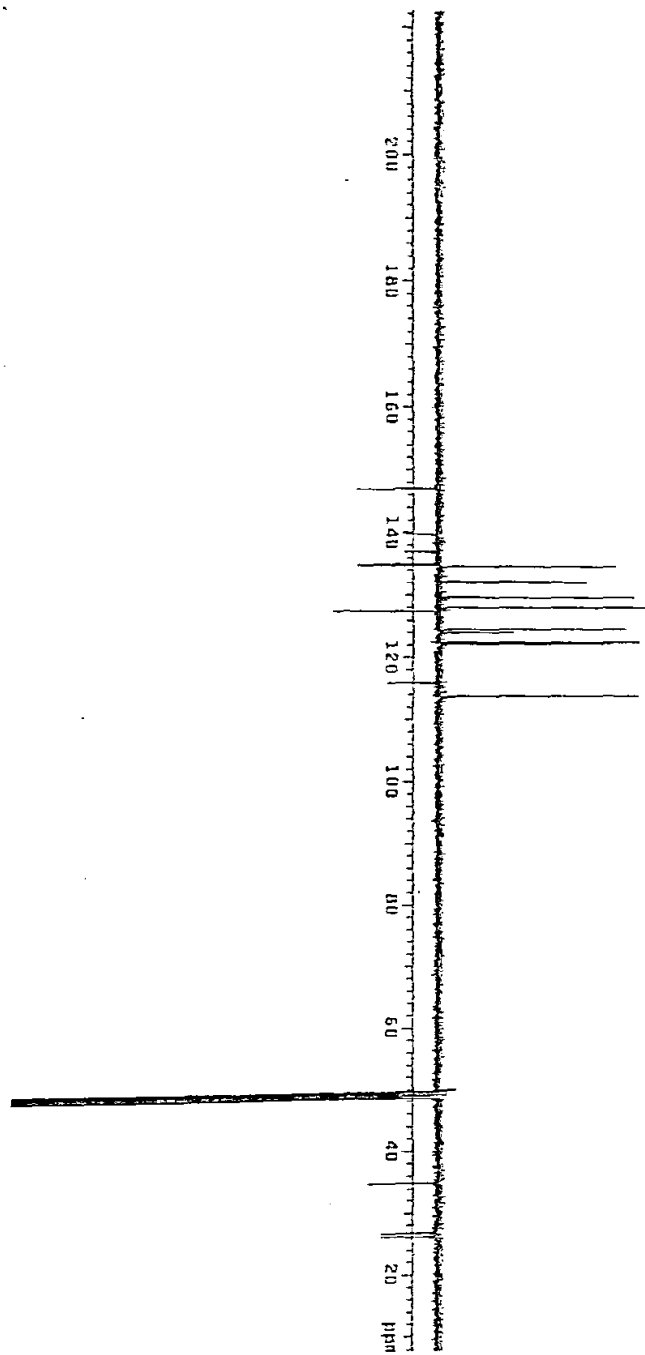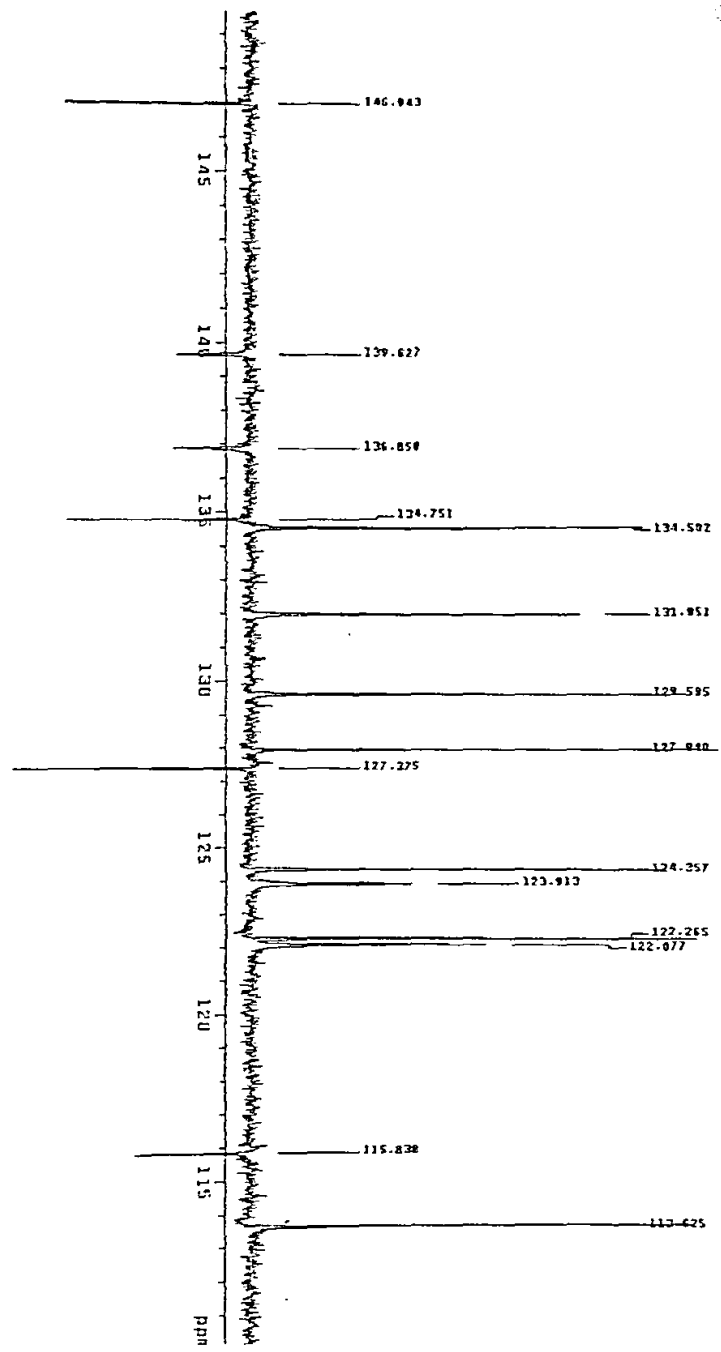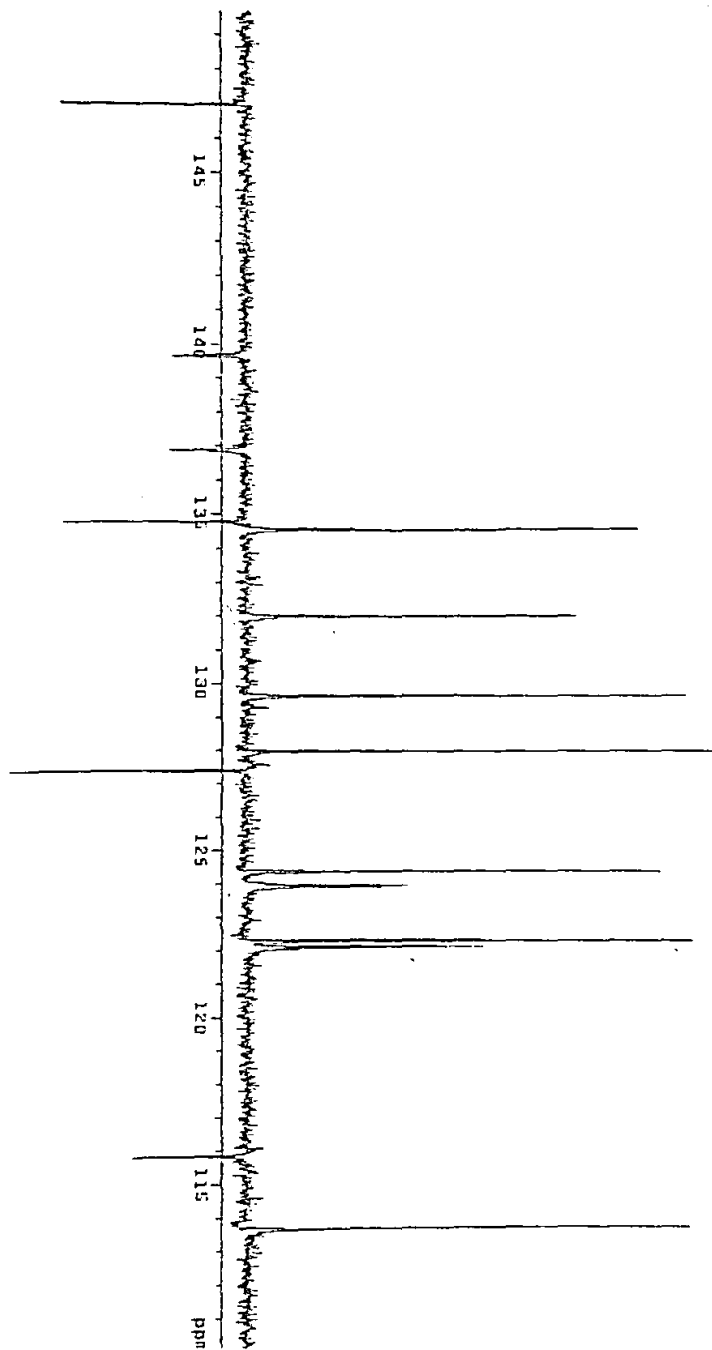
CH4 ~1
500 MHz
CD₃OD
NT = 16

CH4-1/GL

Autospec

07-Jun-2006
12:01:56
Magnet EI+
4.04e4

LR0519 48 (2.523) Cm (33:50)

1H s1 sample H1 in CD3OD DPX2-300 PULSE

9.140

8.766
8.737
8.677
8.646

8.499
8.472

8.214
8.191
8.186
8.160

7.967
7.942
7.919
7.868
7.843
7.815

7.592
7.565
7.541

1.000
2.061
1.023
1.042
2.086
1.226
1.056

1H s1 sample H1 in CD3OD DPX2-300 PULSE

9.140
8.766
8.737
8.677
8.646
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8.214
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7.967
7.942
7.919
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7.815
7.592
7.565
7.541

5.113
4.843
4.562

3.343
3.313
3.308
3.302
3.297
3.292

1.000
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2.086
1.226
1.056
3.310

13C sl sample H1 in CD3OD DMX600

146.332

138.428

135.773

133.956
133.642
132.520

129.650

126.993
126.579
125.372
124.663

121.554

116.837

113.916
112.829

13C s1 sample H1 in CD3OD DMX600

— 146.332
— 138.428
— 135.773
— 133.956
— 133.642
— 132.520
— 129.650
— 126.993
— 126.579
— 125.372
— 124.663
— 121.554
— 116.837
— 113.916
— 112.829

— 78.005
— 77.744
— 77.483

— 63.874
— 48.381
— 48.157
— 48.044
— 47.987
— 47.872
— 47.817
— 47.703
— 47.531
— 47.363
— 47.190
— 47.022
— 39.212

1H COSY s1 sample H1 in CD3OD DMX600

1H NOESY s1 sample H1 in CD3OD DMX600

1H NOESY 51 sample H1 in CD3OD DMX600

1H NOESY s1 sample H1 in CD3OD DMX600

1H COSY s1 sample H1 in CD3OD DMX600

1H/13C HMBC s1 sample H1 in CD3OD DMX600

APPENDIX IX b: CH2

1H/13C HMBC sl sample H1 in CD3OD DMX600

APPENDIX IX c: CH2

1H/13C HMBC s: sample H1 in CD3OD DRX600

1H/13C HMBC s1 sample H1 in CD3OD DMX600

1H/13C HMBC sl sample H1 in CD3OD DMX600