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**CHAPTER – 1**  
**ALKALOIDS, AN OVERVIEW**

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## ALKALOIDS, AN OVERVIEW

Alkaloids are naturally occurring organic substances, predominantly found in plant sources including marine algae and rarely in animals (e.g. in the toxic secretions of fire ants, ladybugs and toads). They occur mostly in seed-bearing plants mainly in berries, bark, fruits, roots and leaves. Alkaloids often contain at least one nitrogen atom in heterocyclic ring (Fig. 1). These are basic in nature and so referred the term alkaloid (alkali-like). Alkaloids possess remarkable physiological action on human and other animals. These are the active components of numerous medicinal plants or plant-derived drugs. Their structural diversity and different physiological activities are unique to any other group of natural products. Many drugs used by man for both medical and non medical purposes are produced in nature in the form of alkaloids e.g. atropine, strychnine, caffeine, nicotine, morphine, codeine, cocaine etc. Naturally occurring receptors for many alkaloids have also been identified in human and other animals, suggesting an evolutionary role for the alkaloids in physiological processes. Alkaloids are relatively stable compounds that accumulate as end products of different biosynthetic pathways, mostly starting from common amino acids such as lysine, ornithine, tyrosine, tryptophan, and others. These substances are usually colourless but several coloured alkaloids are also reported e.g. berberine is yellow, sanguinarine salt is copper-red and betanidin is red (Kokate et al., 2005). These are crystalline solids, having ring structure, definite melting points and bitter in taste. In plants they may exist in free state, in the form of salt or as N-oxides, rarely found in the form of glycosides (Biswas and Sharia, 1978; Tanahashi et al., 2000; Kashiwaba et al., 2000). In addition to the elements carbon, hydrogen and nitrogen, most alkaloids contain oxygen. A few such as coniine from hemlock and nicotine from tobacco are oxygen free. The free bases are sparingly soluble in water but readily soluble in organic solvents, however with their salts, the reverse is often the case, e.g. strychnine hydrochloride is more soluble in water than in organic solvents. Most of the alkaloids are optically active, generally due to the presence of tertiary nitrogen in their structures. This results the various isomeric forms having different physical, chemical and pharmacological properties e.g. (+)-tubocurarine isolated from *Chondrodendron tomentosum* (Bisset, 1992), have muscle relaxant activity, whereas its leavo isomer is less active. The structural formula of some common alkaloids from plant origin are given in Fig. 1 and 2.

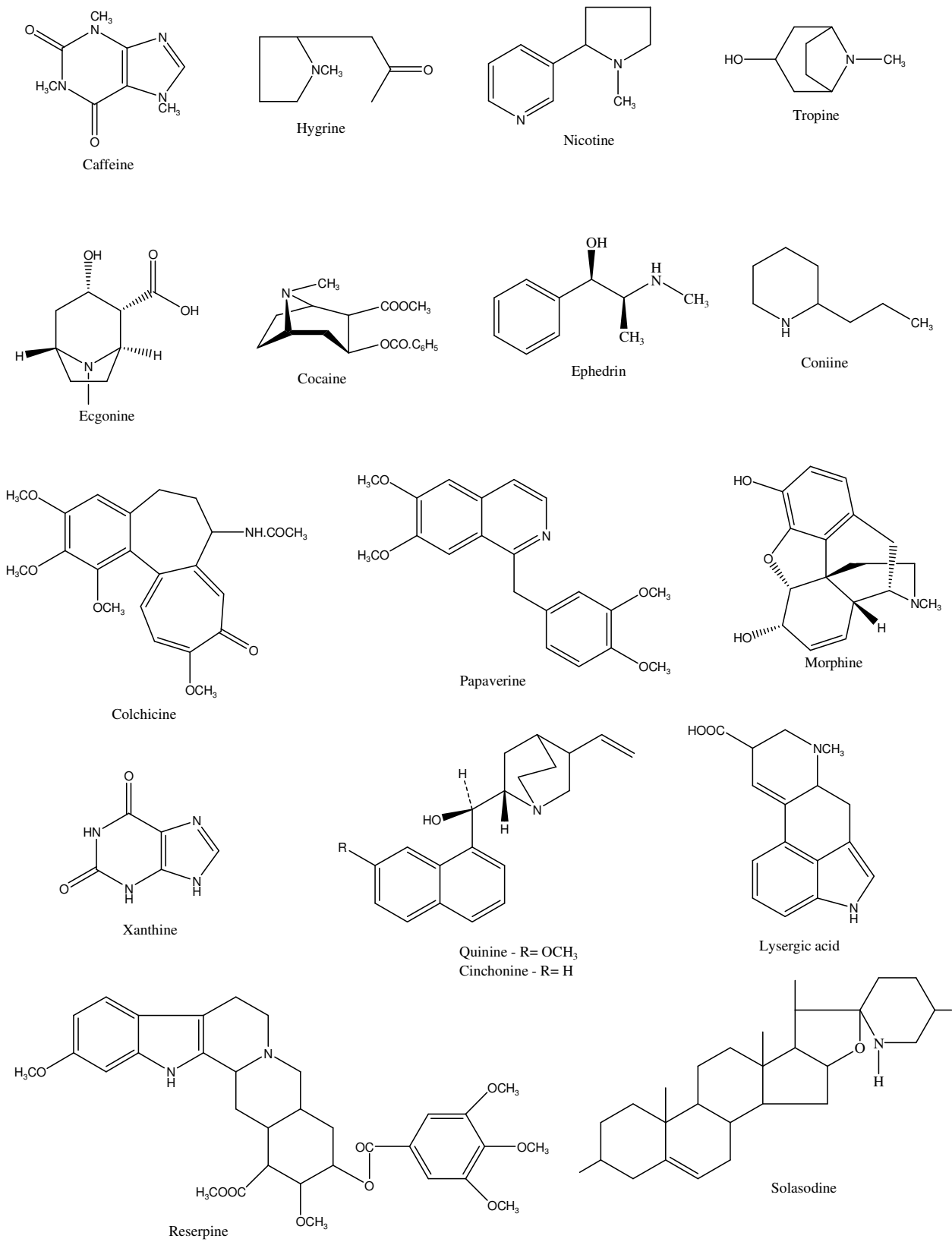


Fig. 1

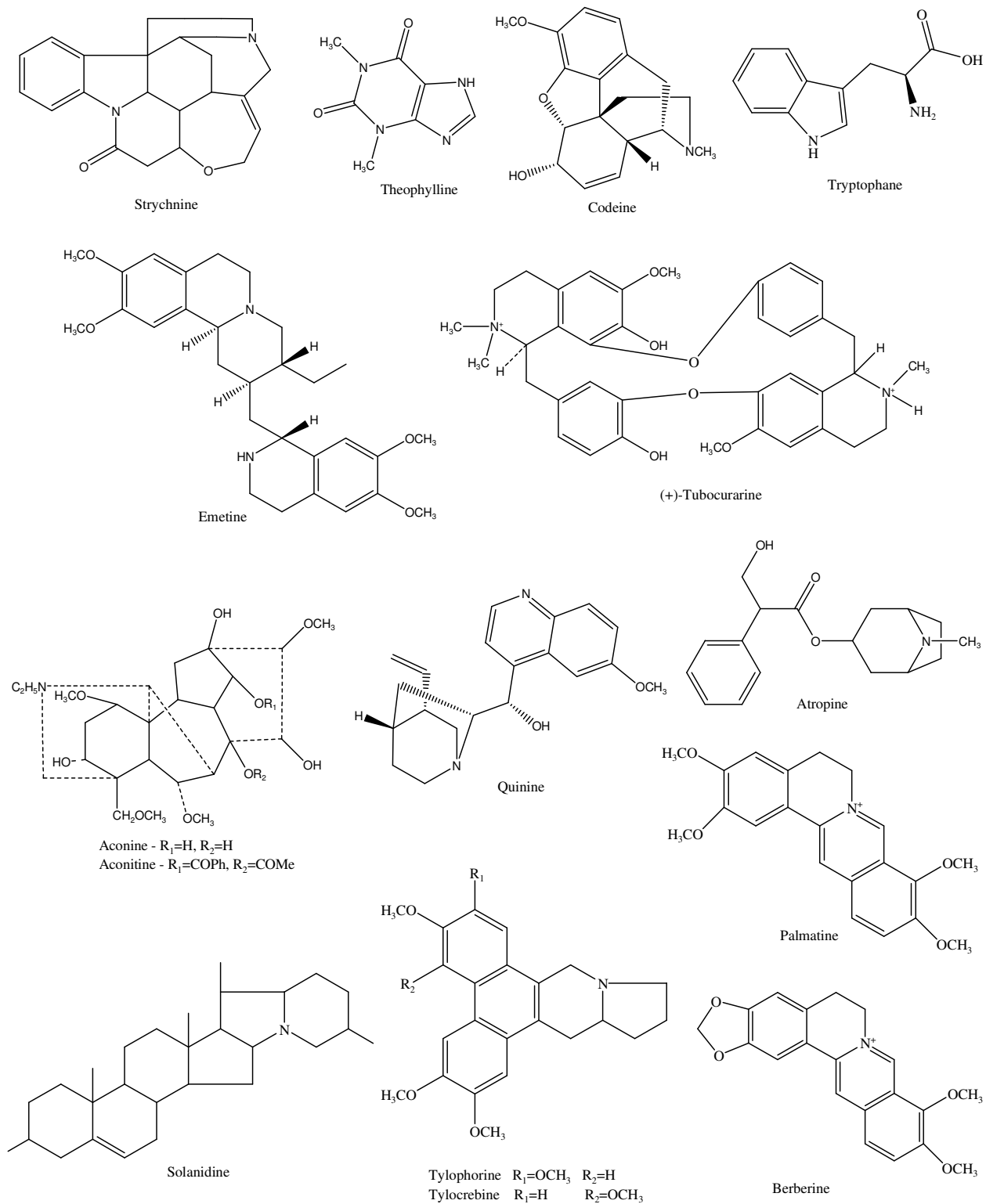


Fig. 2

## **DISTRIBUTION/ OCCURRENCE**

Alkaloids are generally occur in all parts of the plant but some times accumulated only in particular organ, whereas at the same time other organs are free from alkaloids e.g. the edible tubers of potato plant are devoid of alkaloids, whereas the green parts contain the poisonous alkaloid solanine. The organ in which alkaloids accumulated is not always the site of their synthesis, e.g. in tobacco, nicotine is produced in the roots and translocated to the leaves where it accumulates (Harborne and Herbert, 1995).

After the isolation of first alkaloid narcotine by French apothecary Derosne in 1803 and morphine by Hanoverian apothecary Serturner in 1806, more than ten thousand alkaloids have been discovered from different sources (Evans, 2006). Alkaloids are commonly found in the orders Centrospermae, Magnoliales, Ranunculales, Papaverales, Rosales, Rutales, Gentiales, Tubiflorae and Campanulales. True alkaloids are rarely occur in lower plants. Among the Pteridophytes and Gymnosperms, the bioactive alkaloids lycopodium, ephedra and taxus are well known. Lysergic acid and sulphur containing alkaloid gliotoxin are best known examples isolated from fungi.

Nearly 300 alkaloids belonging to more than 24 classes are found in the skin of amphibians along with other toxins (Evans, 2006). The poisonous neurotoxic alkaloids were isolated from the skin of frogs belonging to genus *Phyllobates*. Daly, (1993) isolated various antimicrobial alkaloids from the skin of reptilian. Some indole and isoquinoline alkaloids were isolated from mammals including mammalian morphine.

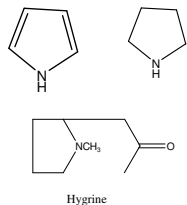
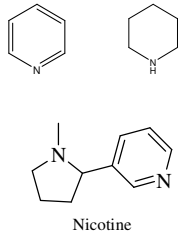
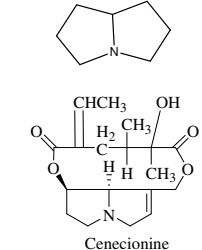
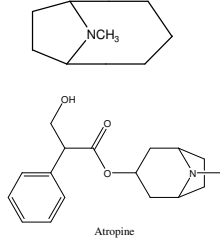
## **CLASSIFICATION OF ALKALOIDS**

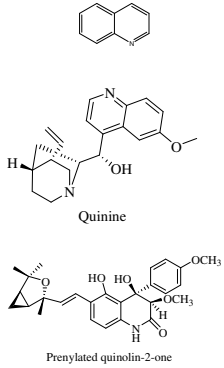
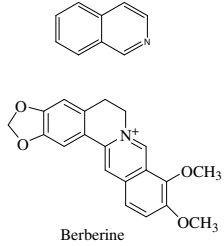
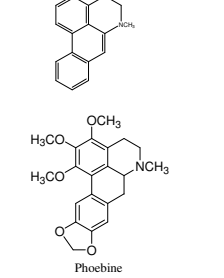
1. **Taxonomical classification:** This classification is based on the distribution of alkaloids in various plant families, like solanaceous or papilionaceous alkaloids. Some times they are grouped as per the name of grouped genus in which they occur, e.g. ephedra, cinchona, etc.
2. **Biosynthetic classification:** This method gives significance to the precursor from which the alkaloids are biosynthesized in the plant. Hence the variety of alkaloids with different taxonomic distribution and physiological activities can be brought under same group, if they are derived from same precursor, e.g. all indole alkaloids from tryptophan are grouped together. Alkaloids derived from amino acid precursor are grouped in same class such as ornithine, lysine, tyrosine, phenylalanine, tryptophan, etc.

3. **Pharmacological classification:** This classification is based on the physiological action or biological activity of alkaloids on animals like CNS stimulants or depressants, sympathomimetics, analgesics, purgatives, etc. This method does not take account of chemical nature of alkaloids. Within the same chemical structure the alkaloids can exhibit more than one physiological action e.g. morphine is narcotic-analgesic, while quinidine is cardiac depressant.
  
4. **Chemical classification:** this classification is most accepted way to specify the alkaloids. The alkaloids are categorised into three divisions.
  - a. **True alkaloids:** These have heterocyclic ring with nitrogen and derived from amino acids.
  
  - b. **Proto alkaloids:** These do not have heterocyclic ring with nitrogen and derive from amino acids, e.g. colchicine.
  
  - c. **Pseudo alkaloids:** These have heterocyclic ring with nitrogen and derived from terpenoids or purines but not derived from amino acids.

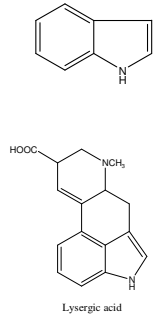
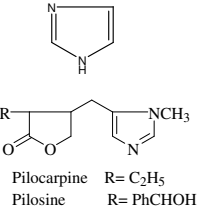
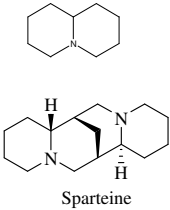
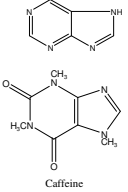
The chemical classification of alkaloids is summarized in Table 1 and a general scheme for classification of alkaloids is shown in Fig. 3.

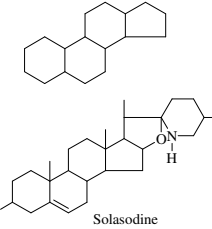
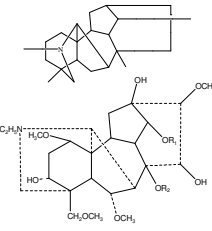
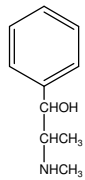
**Table 1.** Classification of alkaloids along with some common examples and sources

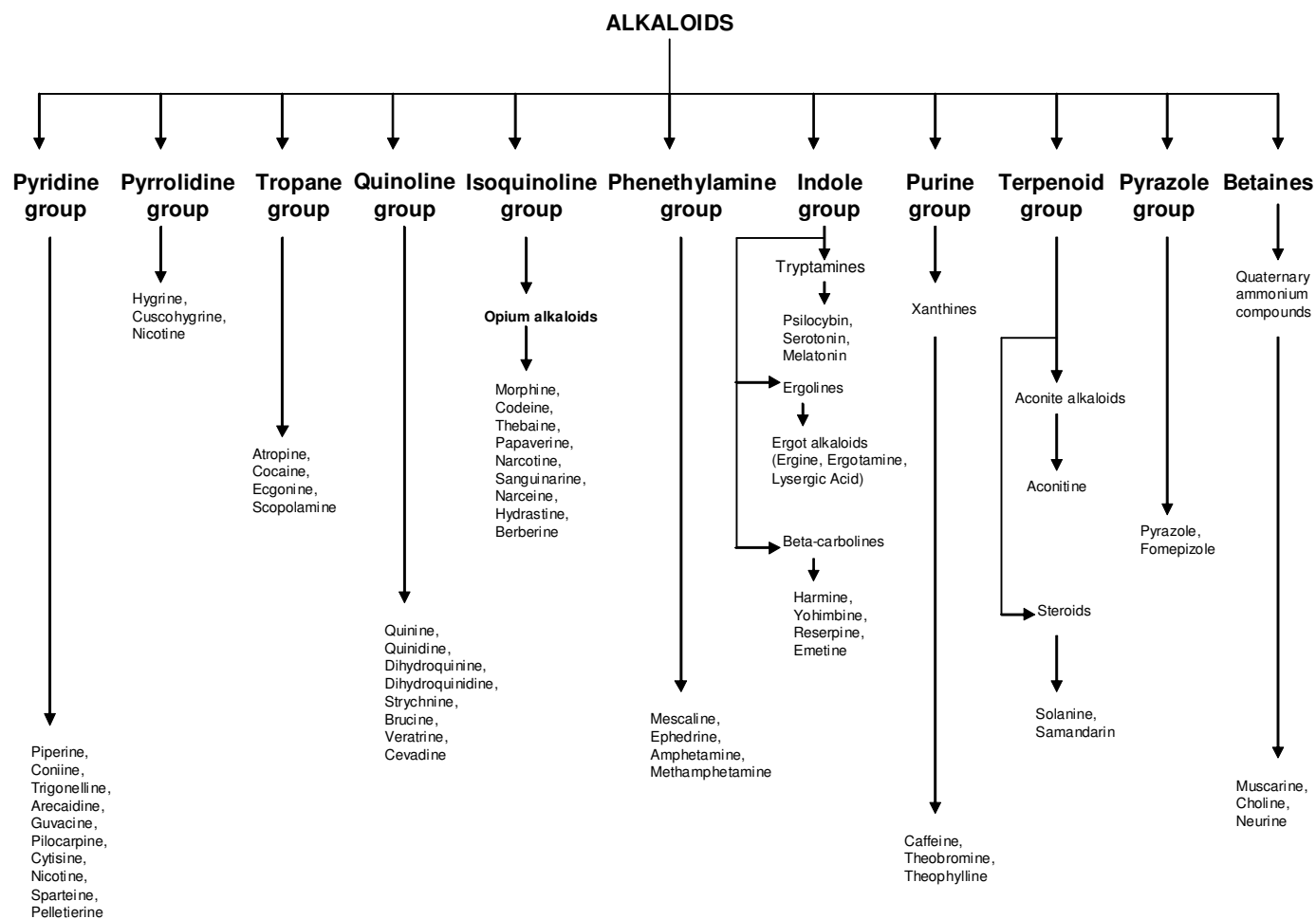
S.No.	Class	Basic ring	Example	Biological sources	References
1.	Pyrrole and Pyrrolidine	 <p>Hygrine</p>	Hygrine, nicotine, cuscohygrine, coca alkaloids	<i>Erythroxylum coca</i> , <i>Erythroxylum truxillense</i>	(Moor, 1994; Evans, 1981)
2.	Pyridine and Piperidine	 <p>Nicotine</p>	Piperine, coniine, trigonelline, arecaidine, guvacine, pilocarpine, cytisine, nicotine, sparteine, pelletierine, lobeline, arecoline, anabasine	<i>Piper nigrum</i> , <i>Areca catechu</i> , <i>Lobelia nicotianefolia</i>	(Parmar et al., 1997; Ravindran et al., 2000)
3.	Pyrrolizidine	 <p>Ceneconine</p>	Echimidine, senecionine, senesiphylline, symphitine	<i>Castanospermum australe</i> , <i>Senecio</i> sps.	(Molyneux, 1988; Hartmann and Witte, 1995)
4.	Tropane	 <p>Atropine</p>	Atropine, cocaine, ecgonine, scopolamine, hyoscine, meteloidine hyoscyamine, pseudo-pelletierine,	<i>Atropa belladonna</i> , <i>Datura stramonium</i> , <i>Erythroxylon coca</i> , <i>Schizanthus porrigens</i>	(Munoz and Cortes, 1998; Leete, 1988; Drager and Schaal, 1993)

5.	Quinoline	 <p>Quinine</p> <p>Prenylated quinolin-2-one</p>	Quinine, quinidine, brucine, veratrine, dihydroquinine, dihydroquinidine, strychnine, cevadine, cinchonine, cupreine, cinchonidine, prenylated quinolin-2-one	<i>Cinchona officinalis</i> , <i>Cinchona calisaya</i> , <i>Almeidea rubra</i>	(Giroud, 1991; Phillipson et al., 1981; Santos et al., 1998; Scherlach and Hertweck, 2006)
6.	Isoquinoline	 <p>Berberine</p>	Morphine, codeine, thebaine, papaverine, narcotine, sanguinarine, narceine, hydrastine, berberine, d-tubocurarine, emetine, cephaeline, narcotine	<i>Papaver somniferum</i> , <i>Cephaelis ipecacuanha</i> , <i>Berberis aristata</i> , <i>Aristolochia elegans</i> , <i>Cocculus pendulus</i>	(Wu et al., 2000; Bisset, 1992; Schiff, 1991; Guinaudeau and Bruneton, 1993; Bhakuni et al., 1976; Uprety and Bhakuni, 1975)
7.	Aporphine	 <p>Phoebine</p>	Boldine, phoebine, laurodionine, noraporphine, norpurpleine, nordelporphine	<i>Stephania venosa</i> , <i>Phoebe valeriana</i>	(Pharadai, 1985; Castro et al., 1986)



8.	Indole	 <p>Lysergic acid</p>	Psilocybin, serotonin, melatonin, reserpine, ergine, vincristine, vinblastine, strychnine, brucine, emetine, physostigmine, harmine, ergotamine, ergometrine, lysergic acid, yohimbine, hydroxygelsamydine	<i>Strychnos nuxvomica</i> , <i>Claviceps purpurea</i> , <i>Rauwolfia serpentine</i> , <i>Catharanthus roseus</i> , <i>Gelsemium elegans</i>	(Dembitsky et al., 2005; Lin, 1996)
9.	Imidazole	 <p>Pilocarpine R= C<sub>2</sub>H<sub>5</sub>          Pilosine R= PhCHOH</p>	Pilocarpine, pilosine	<i>Pilocarpus jaborandi</i> , <i>Maranham jaborandi</i> , <i>Ceara jaborandi</i>	(Negri, 1998)
10.	Norlupinane	 <p>Sparteine</p>	Cytisine, laburnine, lupanine, sparteine	<i>Punica granatum</i> , <i>Lobelia inflata</i>	(Yusuph and Mann, 1997)
11.	Purine	 <p>Caffeine</p>	Caffeine, theobromine, theophylline	<i>Theobroma cacao</i> , <i>Coffea arabica</i>	(Shervington, 1998)

12.	Steroidal	 <p style="text-align: center;">Solasodine</p>	Solasodine, solanine, solanidine, samandarin, protoveratrine, conessine, funtumine	<i>Solanum tuberosum</i> , <i>Veratrum album</i> , <i>Withania somniferum</i> , <i>Holarrhena antidysenterica</i>	(Kumar and Ali, 2000; Shakirov, 1990; Rahman and Chaudhari, 1995; Rahman and Chaudhari, 1996)
13.	Diterpene	 <p style="text-align: center;">Aconine - R<sub>1</sub>=H, R<sub>2</sub>=H Aconitine - R<sub>1</sub>=COPh, R<sub>2</sub>=COMe</p>	Aconitine, aconine, hypoaconitine	<i>Aconitum napellus</i> , <i>Aconitum japonicum</i>	(Rahman and Chaudhari, 1995; Wang et al., 1992)
14.	Alkylamine/ Amino alkaloid		Ephedrine, pseudoephedrine, colchicine, demecolcine	<i>Ephedra gerardiana</i> , <i>Ephedra sinica</i> , <i>Colchicum autumnale</i>	(He et al., 1999)



**Fig. 3.** General scheme for classification of alkaloids

## CHEMICAL TESTS FOR ALKALOIDS

The chemical tests used for detection of alkaloids depend on their character to precipitate with organic acids in the form of their salts. These are also precipitated by the reaction of compounds of heavy metals like mercury, gold, platinum etc. Caffeine and some other alkaloids which are highly water soluble, do not give the tests with usual reagents. Some common reagents, used to the detection of alkaloids are summarized in Table 2.

**Table 2.** Reagents used for the detection of alkaloids

S.No	Name of reagent	Chemical composition	Colour obtained	Example
1	Mayer's reagent	Potassium mercuric iodide solution	Cream	Common
2	Wagner's reagent	Solution of iodine in potassium iodide	Reddish-brown	Common
3	Dragendorff's reagent	Potassium bismuth iodide solution	Reddish-brown	Common
4	Hager's reagent	Saturated solution of picric acid	Yellow	Common
5	Picrolonic acid	Solution of picrolonic acid	Yellow	Common
6	Tannic acid	Solution of tannic acid		Common
7	Murexide test	*Potassium chlorate+HCl+NH <sub>3</sub>	Purple	Caffeine
8	Mineral acids	Phosphotungstic acid, phosphomolybdic acid	Yellow	Colchicine
9	Acidic p-methyl-aminobenzaldehyde	p-Methyl-aminobenzaldehyde and sulphuric acid	Bluish-violet to red	Indole
10	Nitric acid	Dilute nitric acid	Orange-red	Morphine

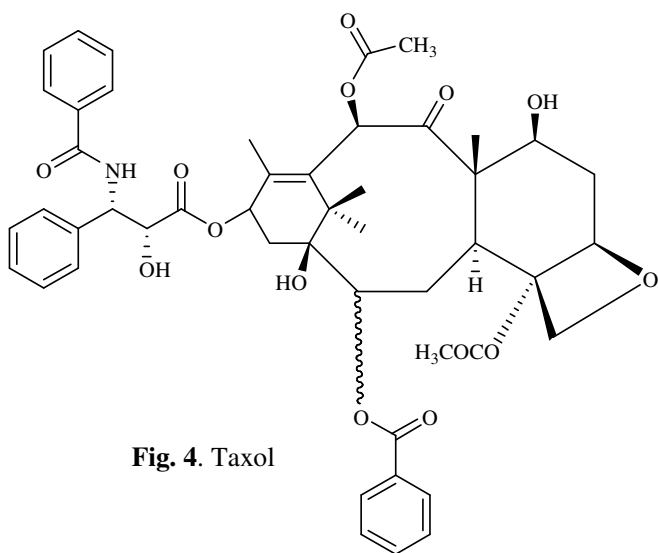
\*a very small amount of potassium chlorate and a drop of conc. HCl, evaporating to dryness and exposing the residue to NH<sub>3</sub> vapour

## PHARMACOLOGICAL ACTION OF ALKALOIDS

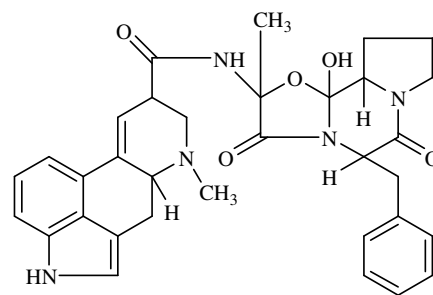
Alkaloids have traditionally been of great interest to humans because of their pronounced physiological and medicinal properties. From the beginning of civilization, alkaloid-containing plant extracts have been used in all cultures as medicines and poisons. Greek philosopher Socrates died in 399 B.C. by consumption of coniine containing hemlock (*Conium maculatum*) and Egyptian queen

Cleopatra (69-30 B.C.) used atropine containing plant extracts (such as belladonna) to dilate her pupils. The physiological effects of alkaloids have made them important compounds in medicine. They are used as a remedy for painkillers, stimulants, muscle relaxants, tranquilizers, anaesthetics, antimalarial, antimicrobial, antidiabetic, anticancerous, anti-HIV, antioxidants etc. Proaporphines and crotsparine isolated from *Cocculus sparciflorus* (Bhakuni et al., 1968), showed significant hypotensive and anticancer activity (Bhakuni et al., 1969). Homoerythrine derived alkaloids isolated from stem of *Galipea bracteata* (Vielra and Kubo, 1990) showed molluscicidal activity. In modern times, the stimulants caffeine in coffee, tea and cacao and nicotine in cigarettes are consumed worldwide. Alkaloids with hallucinogenic, narcotic or analgesic properties have found applications in medicine e.g. morphine, atropine and quinine. Some alkaloids served as model compounds for modern synthetic drugs whereas several are abused as illegal drugs e.g. cocaine. A number of alkaloids are too toxic for any therapeutic use e.g. coniine and strychnine. Moreover the plant constituents are still screened for new biologically active compounds. Taxol (Fig. 4) considered as cytostatic drug, applied for the treatment of lung cancer, breast cancer, neck cancer and ovarian cancer (Heinstein and Chang, 1994). Coniine, tropine, vendoline, morphine and tyrosine showed significant anticancer activity (Hartwell and Abbot, 1969). Nicotine isolated from *Nicotiana tabacum* of family Solanaceae is a highly addictive stimulant of the nervous system in small doses, such as those obtained by smoking tobacco in cigarettes, whereas higher doses can be extremely toxic. Nicotine is also used as an insecticide in the form of nicotine sulfate. Various alkaloids like codeine, heroin, morphine and opium, derived from the sap of *Papaver somniferum* are widely used as medicines or highly addictive and recreational drugs. An addictive narcotic drug cocaine, derived from the foliage of *Erythroxylon coca* used as a stimulant and local anaesthetic (Novak et al., 1984). Quinine, a bitter tasting alkaloid extracted from *Cinchona ledgeriana* is well known for its antimalarial activity. Morphine is a powerful painkiller, often given to terminally ill patients. Codeine having similar pharmacological functions to morphine, but is less potent. Heroin is a synthetic derivative of morphine that is highly addictive. Papaverine isolated from *Papaver somniferum* (Pictet and Gums, 1909) has relaxant effects on smooth muscle of the intestinal and bronchial tract and the blood vessels. Strychnine is a powerful CNS stimulant and lysergic acid produced by a fungus grows on rye in the form of lysergic acid diethylamide (LSD), is a powerful hallucinogen. Atropine (Mann et al., 1994) acts as a smooth muscle relaxant and used to dilate the pupils of the eye. Roots of *Rauwolfia serpentine* used in North India as a sedative, led to the isolation of ajmaline, ajmalinine and serpentine (Siddhiqui and Siddhiqui., 1931). Tylophorine,

tylophoridine and tylocrebine (Hartwell and Abbot, 1969) isolated from different genus of *Tylophora* showed anticancer activity. The bark of *Holarrhena antidysentrica* containing couessine, conessimine and considine (Siddhiqui and Pillay, 1932; 1934) is widely used in the treatment of dysentery. Ergotamine (Fig. 5) in the form of ergotamine tartrate combined with caffeine is used as a specific analgesic in the treatment of migraine.



**Fig. 4.** Taxol



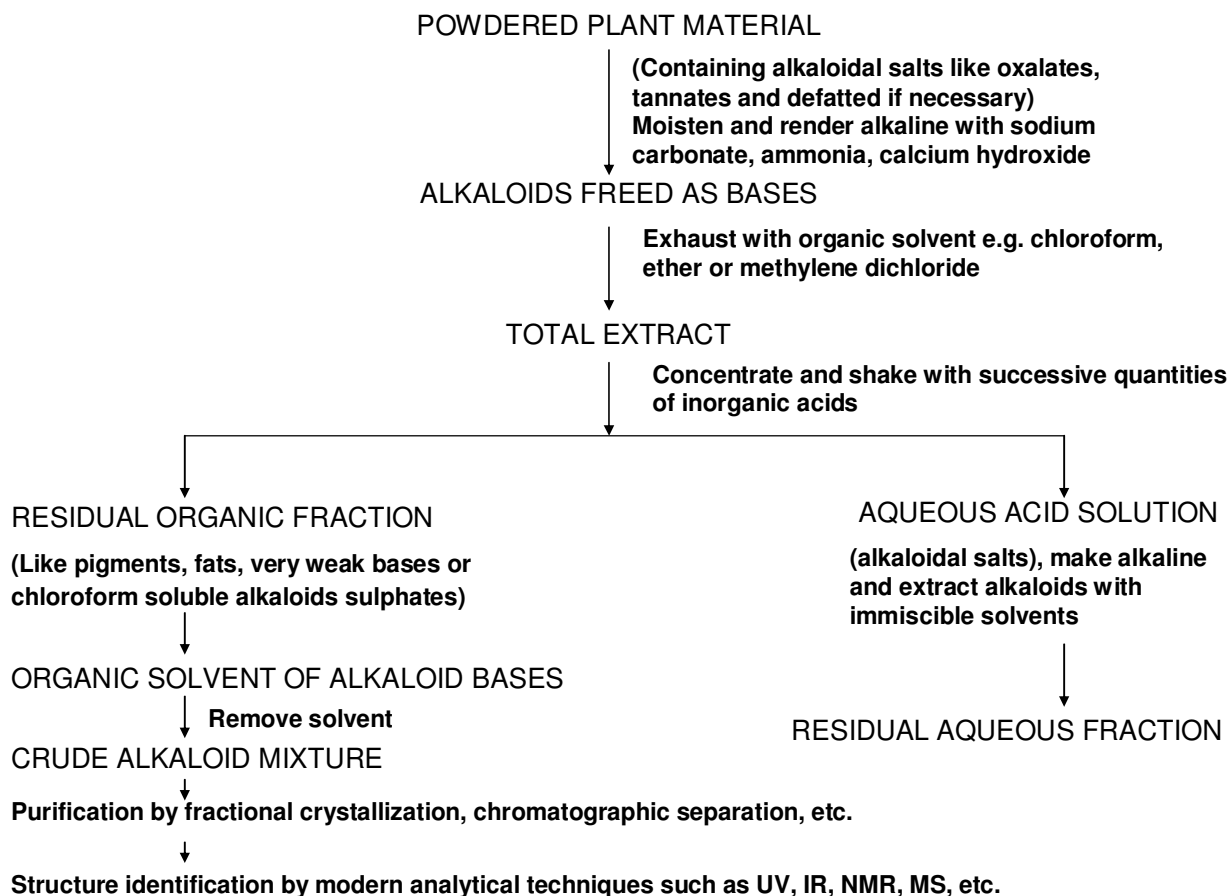
**Fig. 5.** Ergotamine

## EXTRACTION AND ISOLATION

The extraction of alkaloids is based on their basic character and solubility pattern. The general scheme for extraction is shown in Fig. 6. Extraction is usually served by one of the following general methods;

1. The plants are defatted with petroleum ether, especially in case of seeds and leaves to remove the fat soluble constituents and then with polar solvents. The extract is concentrated under reduced pressure and treated with alkali so that the free bases convert in their salts and separated with organic solvents. This process is known as Stas-Otto process. This method is frequently used in the extraction of ergotamine (Kokate et al., 2005) from ergot.
2. The powdered material is moistened with water and mixed with lime, which combines with acids, tannins and other phenolic substances and sets free the alkaloid salts. Extraction is then carried out with organic solvents such as ether or petroleum spirit. The concentrated organic liquid is then shaken with aqueous acid and allowed to separate. Alkaloid salts are now in aqueous liquid, while many impurities remain behind in the organic liquid.

3. The powdered material is extracted with polar solvents such as water or aqueous alcohol containing dilute acid. Pigments and other unwanted materials are removed by shaking with chloroform or other organic solvents. The free alkaloids are then precipitated by the addition of excess sodium bicarbonate or ammonia and then separated by filtration or by extraction with organic solvents.
4. The extract is treated with ammonia so as to convert the alkaloid salts into their free bases. Such liberated alkaloids in free base form are conveniently extracted with organic solvents like ether, benzene, chloroform etc. This method is not useful for the isolation of alkaloids of quaternary nitrogen.
5. The alkaloids present in the extract are converted into their reineckates by treating with  $\text{H}[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4]$  (Reinecke's solution). The product is then dissolves in acetone and then passed this solution through an ion exchange column which afforded the alkaloids in a high state of purity.



**Fig. 6.** General scheme for extraction of alkaloids

Further purification of crude extract of alkaloids is done by following ways, which may, however vary for individual alkaloid.

1. **Direct crystallization from solvent:** It is a simple method of isolation in which the alkaloids crystallise directly by fractionation process and may not be useful in case of complex mixtures.

2. **Steam distillation:** This method is specially employed for volatile liquid alkaloids like coniine, sparteine and nicotine. However this method is not suitable for alkaloids of high molecular weights. In this method, the aqueous extract is made alkaline with caustic soda or sodium carbonate and then alkaloids are distilled off in steam.

3. **Gradient pH technique:** There is variation in the extent of basicity of various alkaloids of same plant. On the basis of this property, the crude alkaloid mixture is dissolved in 2% tartaric acid solution and extracted with benzene so that the first fraction contains neutral and/or very weakly basic alkaloids. The pH value of the aqueous solution is increased gradually by 0.5 increments to pH 9 and extraction is carried out at each pH with organic solvents. By this way, the alkaloids of different basicity are extracted and strongly basic alkaloids extracted at the end.

4. **Chromatographic techniques:** Chromatography is an ideal method for separation of a vast number of alkaloids. The separation of alkaloids carried out by using stationary and mobile phase of different organic solvents. The different techniques of chromatography used for separation of individual alkaloid from complex mixture are as following;

a. **Paper chromatography (PC):** This technique is simplest and most widely used among other chromatographic techniques because of its applicability to isolation, identification and some times quantitative determination of all type of natural products. It is a partition as well as an absorption type technique, in which the mobile phase is either individual or mixture of organic solvents and the stationary phase is hydrophilic surface of paper. The choice of the solvent used to run a chromatogram depends upon the nature of alkaloid. Usually non polar solvents like benzene/ acetone/ water are commonly used for separation of non polar alkaloids and polar solvents like butanol/ acetic acid/ water used for separation of polar alkaloids. Both one and two dimensional ascending and descending, some times horizontal chromatographic techniques applied using Whatman filter paper sheet of various types i.e. No.1, 2, 3 etc. The paper sheet is washed with 5% 2N HCl to remove the impurities before starts the separation. The paper is visualised under UV light



of long wavelength or spread with iodoplatinate reagent in the form of coloured spots. The Rf values and colour appearance of spots on paper sheet are useful in preliminary identification of alkaloids by this technique.

b. **Thin layer chromatography (TLC):** TLC is an important tool extensively used for identification, separation and determination of the purity of isolated alkaloid. Although TLC method is used in qualitative analysis but it having a great importance in quantitative analysis also (Oswald and Fluck, 1964; 1964). The TLC separation of alkaloids can be performed on silica gel, alumina, cellulose powder or kieselguhr. Silica gel is the most active stationary phase and good separation is achieved even when several milligrams substance applied. This method is also used for preparative separation of alkaloids (Tschesche et al., 1963). In TLC, the substances are identified preliminary on the basis of their mobility in suitable solvent system and moreover on the basis of their reaction with selective or specific detection reagents or on the basis of their absorption or fluorescence in UV light (Macek, 1972; Hais, 1970; Neher, 1970). Some typical solvent systems (Stahl, 1969) are as following;

$C_6H_6/ EtOAc/ (C_2H_5)_2NH$ (5:4:1) + 8% MeOH	$C_6H_6/ EtOAc/ (C_2H_5)_2NH$ (5:4:1, 7:2:1)
$CHCl_3/ (C_2H_5)_2CO/ (C_2H_5)_2NH$ (7:2:1) + 8% MeOH	$CHCl_3/ (C_2H_5)_2CO/ (C_2H_5)_2NH$ (7:2:1)
$HCON(CH_3)_2/ (C_2H_5)_2NH/ EtOH/ EtOAc$ (1:1:6:12)	$CHCl_3/ MeOH/ AcOH$ (6:1:3)
$C_6H_{12}/ (C_2H_5)_2NH$ (9:1 or 1:1)	$C_6H_6/ CHCl_3/ (C_2H_5)_2CO$ (14:3:3)
$CHCl_3/ EtOH$ (9:1 or 8:2)	$CHCl_3/ (C_2H_5)_2O/ H_2O$ (7:1:2)
Xylene/ BuOH/ MeOH/ $(C_2H_5)_2NH$ (5:5:7:3)	$C_6H_{14}/ CCl_4/ (C_2H_5)_2NH$ (5:4:1)

Developed TLC plate is sprayed by several visualizing reagents for detection of alkaloids. Many alkaloids can be seen on the chromatogram even in daylight and a large number yield typical fluorescence colours in UV light (365 nm). The reagents most commonly used for detecting alkaloids are Dragendorff reagent, acidified iodine- iodine solution, iodoplatinate, antimony-(III)chloride, cerium sulphate in sulphuric acid and cerium sulphate in phosphoric acid. Papaverrubine type alkaloids give a red spot with hydrogen chloride vapour, *Rauwolfia* alkaloids give colour with mixture of ferric chloride and perchloric acid and also with iodine vapour, phenylalkylamines are visualised with ninhydrin. Beside these reagents, cinnamaldehyde hydrochloric acid for indole alkaloids, sulphuric acid for purine bases and betaines and the van Urk reagent for ergot alkaloids are often used.

**Preparative TLC:** In this method, comparatively thicker layer of adsorbent are employed for preparative work and the separated bands of compounds are scraped from the plate and subjected to solvent extraction. This technique generates sufficient quantity of material for complete spectral analysis. Preparative TLC was performed for cryptopleurine and piperidylacetophenone (Al-Shamma et al., 1982) on 1 mm thick commercial plates precoated with  $\text{Al}_2\text{O}_3$  GF-254 developing with ammoniated  $\text{CHCl}_3$  and determined the  $R_f$  values.

c. **Column chromatography (CC):** This method is very popular and used extensively for quantitative separation and purification of different natural products from crude plant extract or mixture. Column of different size and dimensions are used for separation. Choice of stationary phase (adsorbents) and mobile phase (organic/ inorganic solvents) is based on the nature of components present in the crude extract. A large number of adsorbents are available which can be used in the column chromatography, some being better for one type of components and some for others. For the separation of alkaloid, silica gel (Yinggang et al., 2003; Roumy et al., 2006) and neutral alumina (Hart et al., 1968) are extensively used. Beside these, some more adsorbents are kieselguhr, silicates (magnesium/calcium silicate), tricalcium phosphate, calcium sulphate, glass power, salts of heteropoly- tungstic, molybdic and tetraboric acids, ferric and chromic oxides, zinc carbonate/ ferrocyanide, charcoal, zirconium phosphate, hydrous zirconium oxide, lanthanum oxide, bentonites, cellulose powder (including acetylated, ion exchanger), starch, sucrose, mannitol, dextran gels (sephadex), polyamides etc. Eluotropic solvents used in column chromatography, in order of increasing polarity are petroleum ether, n-hexane, cyclohexane, carbon tetrachloride, trichloroethylene, benzene, dichloromethane, chloroform, diethyl ether, diethyl formamide, ethyl acetate, pyridine, n-propanol, ethanol, methanol, formamide and water. Polarity and composition of solvents is depending upon the nature of compound to be eluted i.e. non-polar compounds elutes with non-polar solvents whereas polar compounds elutes with polar solvents. Polarity is changed by observing the mobility of the compounds to be elutes. Some solvent systems are commonly used for the separation of alkaloids are as follows;

Petroleum ether- chloroform (1-100%), n-hexane- chloroform (1-100%), benzene- chloroform (1-100%), methanol- chloroform (1-100%), methanol- ethyl acetate (1-100%), pet. ether- ethyl acetate- diethylamide (5:1:0.3-20:1:0.3)

d. **Gas liquid chromatography (GLC):** Gas chromatography separates volatile substances by percolating a gas stream over a stationary phase. It can be applied to almost any type of compound

which has reasonably volatility and stable under the chromatographic conditions. GLC is the most selective and versatile form of gas chromatography, since there exists a wide range of liquid phases usable upto 450<sup>0</sup>C. This method is applicable for the examination of alkaloids of opium, tobacco, coniine and belladonna. Modification of the sample structure may be accomplished by derivatisation and the most widely applied general derivatisation is silylation. The compound containing labile functional groups viz. alcohols, amines, carboxylic acids and thiols are converted into corresponding silylamines, esters and thioesters. The common derivatising agents used in GLC are trimethylchlorosilane, hexamethyldisilazone and N, O-bis-trimethylacetamide.

e. **High performance thin layer chromatography (HPTLC):** This method is very useful in both qualitative and quantitative analysis. It is a major advancement of TLC, which requires shorter time, saving reagents and better resolution. The basic difference between conventional TLC and HPTLC is only in particle and pore size of the sorbents. The linear development method is most familiar technique in HPTLC. The analytical profiles for tropine alkaloids and several other natural products i.e. flavonoids and steroids have been developed using this technique. HPTLC is conveniently applied for compilation of profiles pertaining to varied range of bioactive constituents such as berberine, quinine, opium alkaloids, colchicines, chelidone, sanguinarine, serpentine, raubasine, asarone, elemicine, eugenol, thymole coumarine, pulegon, flavones, diosgenin, silymarin, catechin, anthraquinone derivatives, gibberellins, antibiotics and number of other compounds of natural origin. Using external standards, the detection limits of bulaquine, chloroquine, and primaquine by HPTLC (Saxena et al., 2003) on silica plates were 0.25, 0.59 and 0.53 µg respectively while the lower limits of quantification were 0.52, 1.21 and 1.07 µg respectively with detection wavelength at 254 nm. The separation was achieved on precoated Silica gel 60 F254 (20×20 cm, 0.2 mm) plates using n-hexane/ diethyl ether/ methanol/ diethylamine (37.5:37.5:25:0.5, v/v) as solvent system. HPTLC is a sophisticated and automated form of TLC.

f. **High performance liquid chromatography (HPLC):** In HPLC method, the separation of one component from another is based upon their absorption of UV light and the functional group interaction with the analytical column substrate. The amount of caffeine in a sample of coffee may be determined to a high degree of accuracy using HPLC techniques. It has become the most versatile, safest, dependable, fastest and sensitive chromatographic technique for the quantitative and qualitative analysis of natural as well as synthetic compounds. The alkaloids like morphine, papaverine, codeine, emetine, antibiotics, ergot alkaloids etc. can be analysed by HPLC. Since the

time of its invention in 1966 by Horvath and Lipsky, several natural products including several antibiotics (Cooper et al., 1973; Buhs et al., 1974; Wold et al., 1977) has been determined qualitatively as well as quantitatively successfully. Chloroquine, primaquine and bulaquine has resolved (Saxena et al., 2003) with the help of HPLC without no interference with retention times of 4.22, 5.72 and 17.26 min respectively by using RP C8 lichrospher 25 x 0.4 cm, 5 $\mu$  column and the solvent was acetonitrile: sodium acetate buffer (pH 5.6) in ratio of 55:45 with detection wavelength 265 nm. The profile of berberine bridge enzyme catalyzed transformation of (S)-reticulon to (-)-scoulerine (Winkler et al., 2006) was carried out at four different time to determine the reaction progress. The series of common solvents used in this technique with relative decreasing order of polarity is acetic acid, ethylene glycol, glycerol, formamide, acetic anhydride, furfural, acetonitrile, methanol, ethanol, dimethyl formamide, propanol, iso-propanol, methyl ethyl ketone, butanol, cyclohexanol, pyridine, diethyl sulfoxide, methyl acetate, ethyl acetate, dioxane, tetrahydrofuran and chloroform.

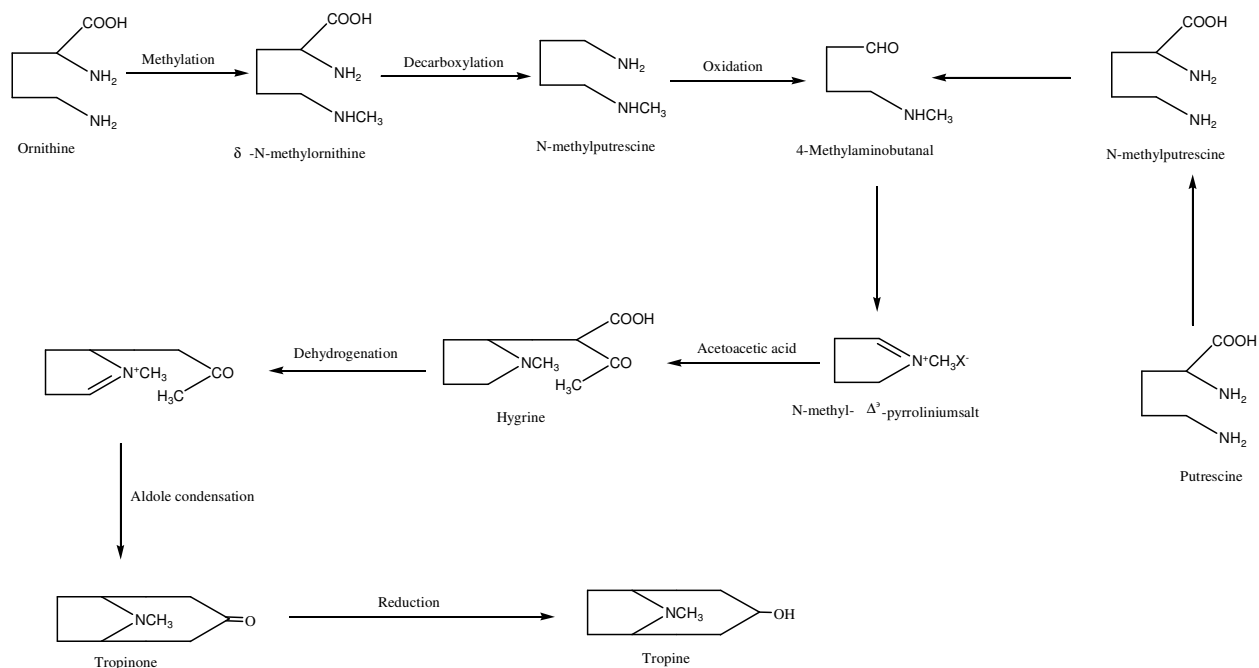
## **ROLE OF ALKALOIDS IN PLANTS**

The functions of alkaloids in plants are mostly unknown and their importance in plant metabolism has been debated. A single plant species may contain over one hundred different alkaloids and their concentration can vary from a small fraction to as much as 10% of the dry weight. Most alkaloids are very toxic and therefore, have the potential function in the chemical defence arsenal of plants against attack by herbivores and micro-organisms (Levin, 1976; Levin and York, 1978). For example, nicotine present in tobacco leaves inhibits the growth of tobacco hornworm larvae. Nicotine in pure form is also applied as an effective insecticide in greenhouses. In addition, alkaloids have been suggested to serve as a storage form of nitrogen or as protectants against damage by ultraviolet light. Some Phytochemists suggested that alkaloids are by-products of normal plant metabolism. It has been suggested that alkaloids may have a role in the defence of the plant against singlet oxygen ( $^1O_2$ ), which is damaging to all living organisms and is produced in plant tissues (Larson and Marley, 1984; Larson, 1988) in presence of light. It is also thought that alkaloids may provide a means of defence against insects and animals. Alkaloids may also be a reservoir (Wink and Michael, 1999) for molecules that plants often use.

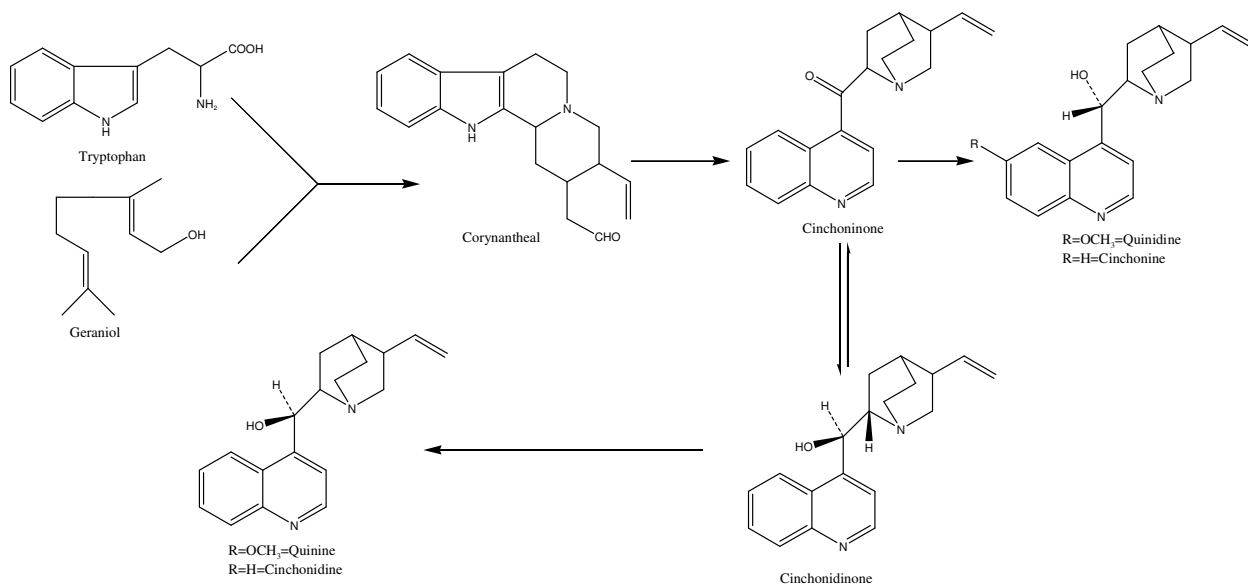
## **BIOSYNTHESIS/ BIOGENESIS OF ALKALOIDS**

The molecular structure of the alkaloids along with other substances occurring with them led to some formulations by which the biosynthesis of most of the alkaloids is studied. Biosynthesis of

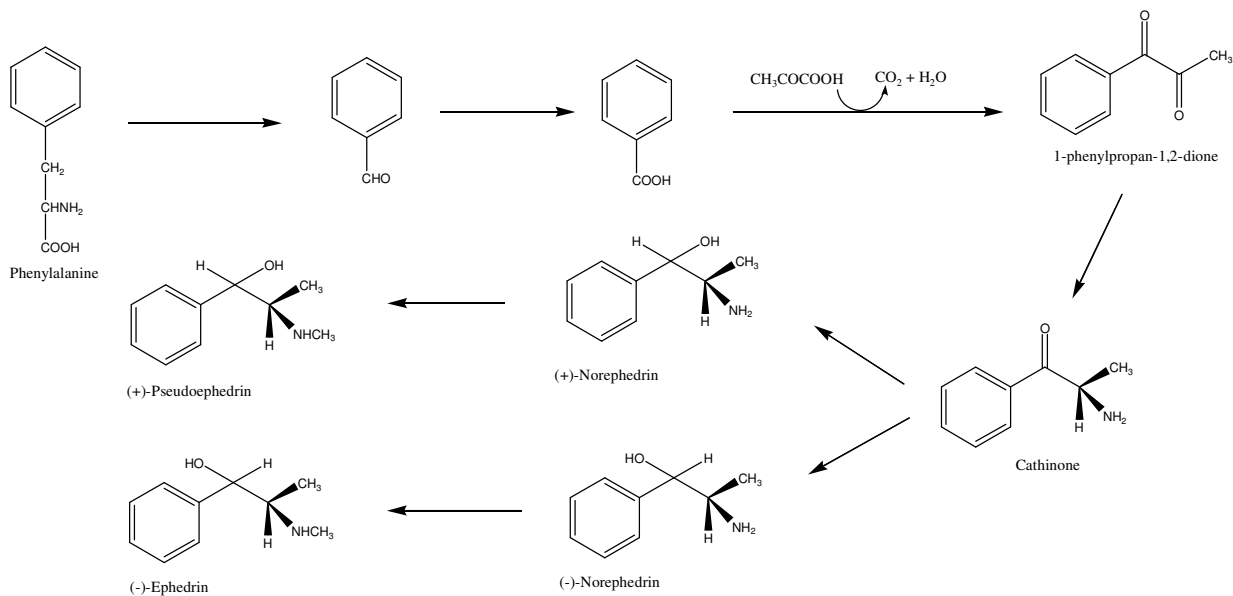
different group of alkaloids has now been investigated to some extent using precursors labeled with radioactive atoms. Ornithine is precursor or starting compound of the synthesis of tropine alkaloids (Lounasmaa and Tamminen, 1993; Patron, 2000) and the intermediate produced during this synthesis is methylornithine (Fig. 7). Tryptophan is starting compound for the biosynthesis of quinoline alkaloids (Fig. 8) (Isaac, 1987). Phenylalanine is starting product in most of the amino acid derived alkaloids like ephedrine (Fig. 9). Tyrosine (Fig. 10) is the starting product of a large family of alkaloids including isoquinoline (Guinaudeau and Bruneton, 1993; Hansine, 1984). The first important intermediate is dopamine which is the starting product of the biosynthesis of colchicine and related alkaloids (Fig. 11). Tryptophan and mevalonic acid are starting precursors in the biosynthesis of indole alkaloids (Fig. 12). The biosynthetic pathways for pharmacognostically important alkaloids are given in Fig. 7-12.



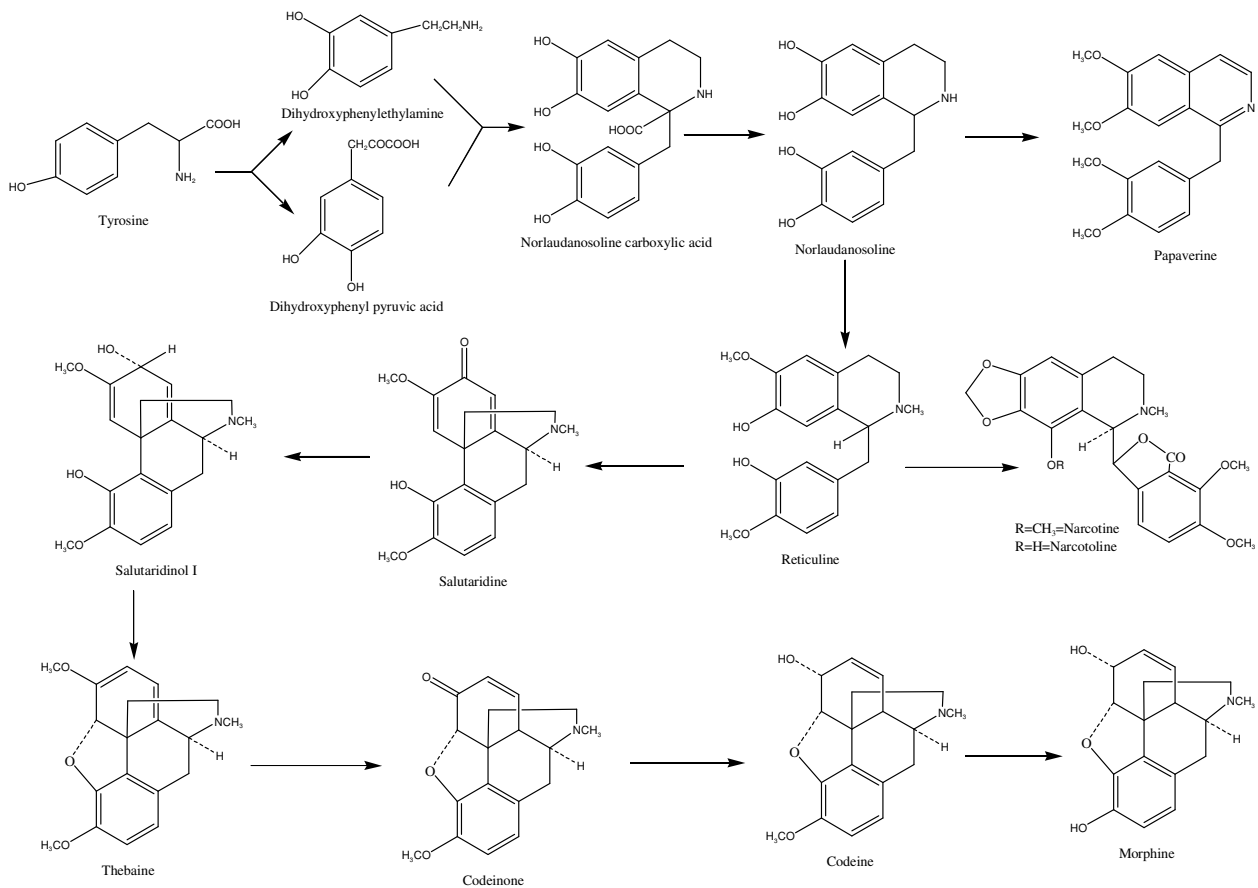
**Fig. 7.** Biogenesis of tropine and pseudotropine alkaloids



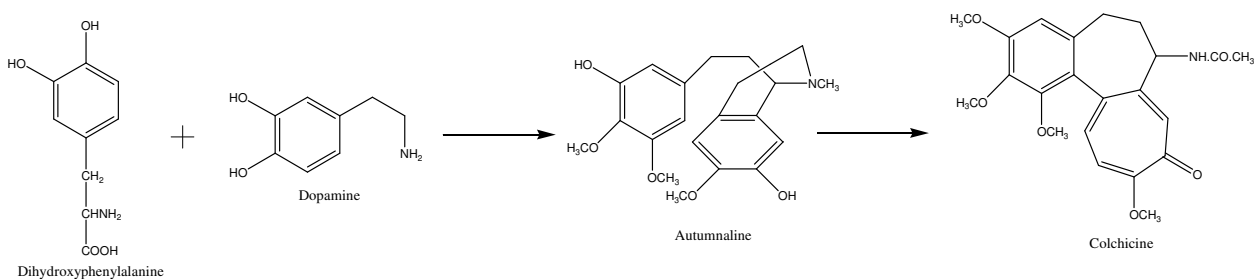
**Fig. 8.** Biogenetic pathway of quinoline alkaloids



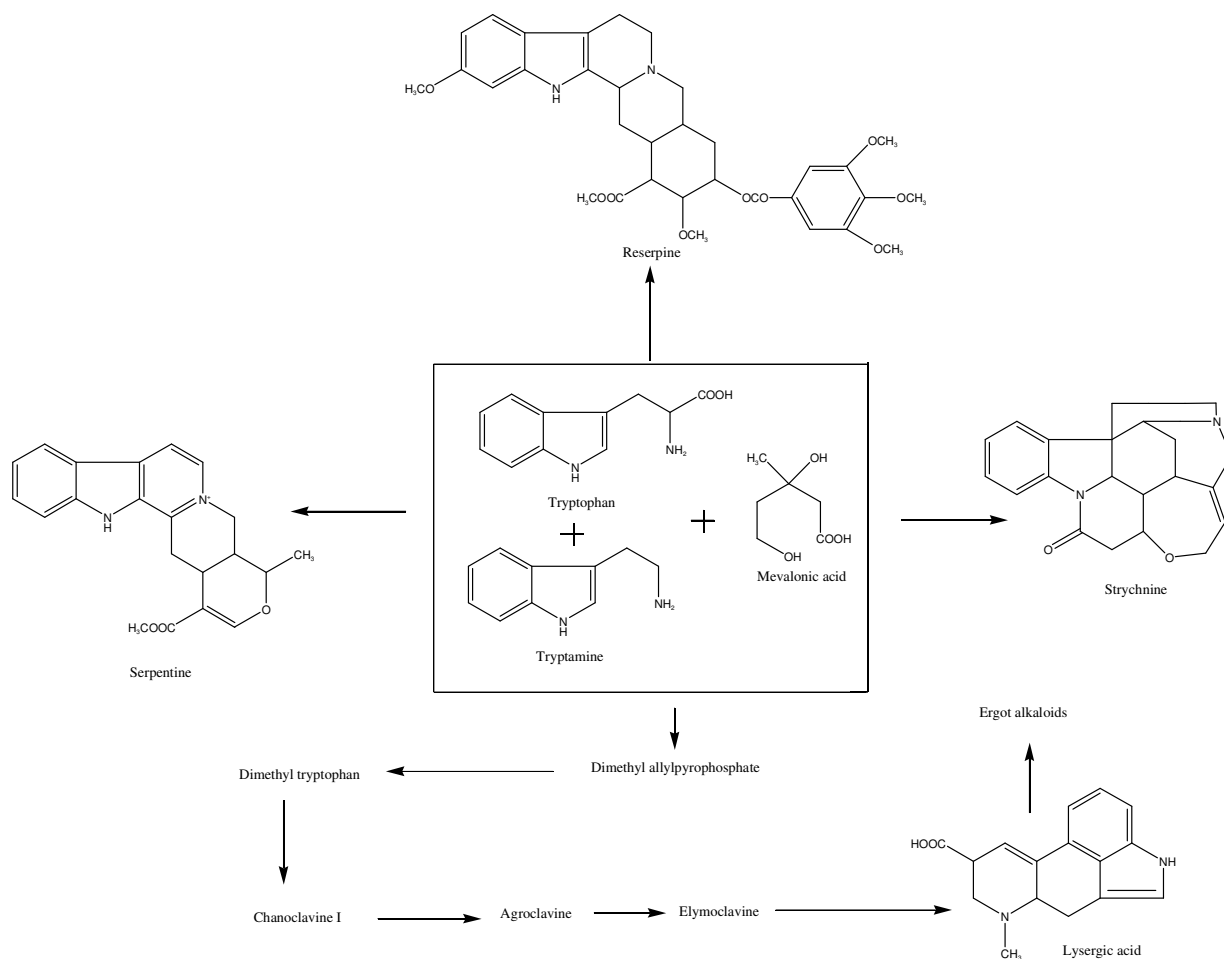
**Fig. 9.** Biogenesis of amino alkaloid (ephedrine and related alkaloids)



**Fig. 10.** Biosynthesis of isoquinoline alkaloids



**Fig. 11.** Biosynthesis of colchicine and related alkaloids



**Fig.12.** Biosynthesis of indole alkaloids

## CHARACTERIZATION OF ALKALOIDS

Spectroscopic studies have revealed important informations regarding the structure of alkaloids. UV, IR, NMR, MS including X-ray crystallography and ORD methods played a significant role in the structure elucidation of natural products. Various modifications of NMR and MS experiments have become of growing importance for the structural characterization of alkaloids.

### UV-Visible spectroscopy

This valuable tool is most frequently used in the identification of structure, especially the chromophore groups including conjugated double bonds present in the molecule. This analytical method is based upon measurement of light absorption by substance in the wavelength of 190-800nm. The region from 190 to 380 nm is known as UV region whereas upto 800 nm (some times 900 nm) is known as visible region. A variety of natural products of pharmaceutical significance



have been analysed and studied by UV-VIS spectroscopic technique. Some of the typical examples are summarized in Table 3.

**Table 3.** Absorption maxima of some common alkaloids

S. No.	Alkaloid	Wavelength ( $\lambda_{\max}$ ) in nm
1	Lobeline	249
2	Reserpine	268, 390 (with sodium nitrite in dilute acid)
3	Vinblastine	267
4	Vincristine	267
5	Tubocurarine chloride	280
6	Morphine	286, 442 (after nitroso reaction)
7	Colchicine	350
8	Ergot (total alkaloids)	550 (with <i>p</i> -dimethylaminobenzaldehyde reagent), 532 (with vanillin in concentrate HCl)
9	Tropic acid, esters of hydroxytropanes	555 (with fuming nitric acid in methanolic KOH)
10	Cardioactive glycosides	217, 590 (after Keller-Kiliani reaction)
11	Vaniline	301
12	Anthraquinone	505 (after treatment with alkali)
13	Indole chromophore	224, 277 (Mukhtar et al., 1997)
14	Aporphine	282, 312 (Castro et al., 1986)

### Infra-red spectroscopy

IR spectrometric studies have proved a very important analytical tool for establishing the presence of certain functional groups in alkaloids. IR spectroscopy is the study of the reflected, absorbed or transmitted radiant energy in region from 0.8 to 500  $\mu\text{m}$ . A more commonly used measurement for IR spectroscopy is the frequency, expressed in wave number. The quantitative analysis of alkaloids, quinine and strychnine (6.20 and 6.06  $\mu$ ) is also possible. FTIR is a new method of spectroscopy which has come into use more recently. It is especially useful for examining small samples and for taking the spectrum of compounds produced in the out flow of a chromatograph. The regions in which functional groups absorb are summarized in Table 4.

**Table 4.** IR absorption regions of some groups

S. No.	Functional groups	Appearance of band (cm <sup>-1</sup> )	S. No.	Functional groups	Appearance of band (cm <sup>-1</sup> )
1	*C-H (str.)	3300-2800	6	C-N (aliphatic) C-N (aromatic)	1210-1200 1350-1250
2	O-H (str.)	3700-3350	7	C≡C, C≡N (str.)	2350-2000
3	C-O (str.)	1280-1000	8	C=C, C=N, N=O (str.) and N-H (bend.)	1640-1500
4	C=O (str.)	1950-1640	9	Double bond (bend.)	950-750
5	#N-H (str.)	3500-3300	10	Other str., bend. and combination bands	1400-400 (finger print region)

\*Saturated acyclic and cyclic hydrocarbons C-H (str.) = 2960-2850, C-H (in plane bend.) = 1470-1360; Unsaturated olefinic C-H (str.) = 3090-3000; Unsaturated acetylenic C-H (str.) = 3300-3270; Aromatic C-H (str.) = 3100-3000, C-H (out of plane bend.) = 900-650.

#NH<sub>3</sub> ≈ 3200; -NH<sub>2</sub> ≈ 2700; >NH ≈ 2000

### Fluorescence spectroscopy

All the molecules absorb light usually over a specific range of wavelength and many of them re-emit such radiations. This phenomenon is called as luminescence. When the re-emission of absorbed light lasts only whilst the substance is receiving the exciting rays, the phenomenon is called as fluorescence. The leaf and root of belladonna, wild cherry bark, gambier catechu aloes, jalap, etc. show fluorescence in visible range. The UV light obtained from mercury vapour lamp or tungsten produce fluorescence in many natural products, which do not visibly fluoresce in day light. Fluorescence produced by a compound in UV light for quantitative evaluation is possible with the help of an instrument spectrofluorimeter. Sennoside, extracts of rhubarb, berberis, and cinchona alkaloids may be evaluated by this technique. Under fluorescent light alkaloids or source of alkaloids are evaluated qualitatively and shows fluorescence is summarized in Table 5.

**Table 5.** Color of fluorescence shown by some common alkaloids and other sources

S. No.	Alkaloid or source of alkaloids	Color of fluorescence
1	Cinchona bark	Luminous yellow and light blue
2	Calumba	Yellow, dark green (phloem and cambium)

3	Hydrastis rhizome	Golden yellow
4	<i>Rheum emodi</i>	Brown
5	<i>Rheum rhaponticum</i>	Violet
6	Ergot powder	Red
7	Olive oil	Deep golden yellow
8	Arachis and sesame oils	Blue
9	<i>Berberis</i>	Yellow
10	Gentian	Light blue
11	Aconitine	Light blue
12	Emetine	Orange
13	Precipitated chalk	Greenish-violet

### Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy was discovered in 1946 and after a number of modifications, it has been routinely applied in organic chemistry since 1960. It is an extremely important tool for elucidation of molecular structure, especially the stereochemistry and configuration of chemical compounds. This technique reveals the position and environment of proton and carbon (some times other nuclei i.e. phosphorus, fluorine etc.) in a complex molecule. It is an excellent choice for qualitative and quantitative analysis of new compounds isolated from natural sources. The advent of 1D and 2D techniques as DEPT, COSY, NOESY, 2D *J*-resolved, one-bond and long range hetero-COSY/COLOC, 2D-INADEQUATE, HOHAHA etc. (Rahman, 1986; Rahman, 1989) have provided the organic chemist with new powerful tools for structure elucidation of complex natural products. Inverse measurement techniques such as HMBC and HMQC have allowed substantial enhancements of sensitivity in the heteronuclear shift correlation experiment. A number of homonuclear and heteronuclear 2D NMR techniques have been developed for identification of alkaloids, for example anopterine alkaloid (John et al., 1985) was identified on the basis of modern 2D NMR analysis. Rahman and Qureshi, (1990) summarized a review on the application of modern 2D experiments for structure elucidation of various new alkaloids. The  $\delta$  values for any particular compound show a discrepancy with deuterated solvent to solvent (Breitmaier and Voelter, 1987; Derome, 1988; Williams and Fleming, 2004).  $^1\text{H}$  and  $^{13}\text{C}$  NMR chemical shifts ( $\delta$  values) of some alkaloids and heterocyclic compounds are given in Fig. 13.

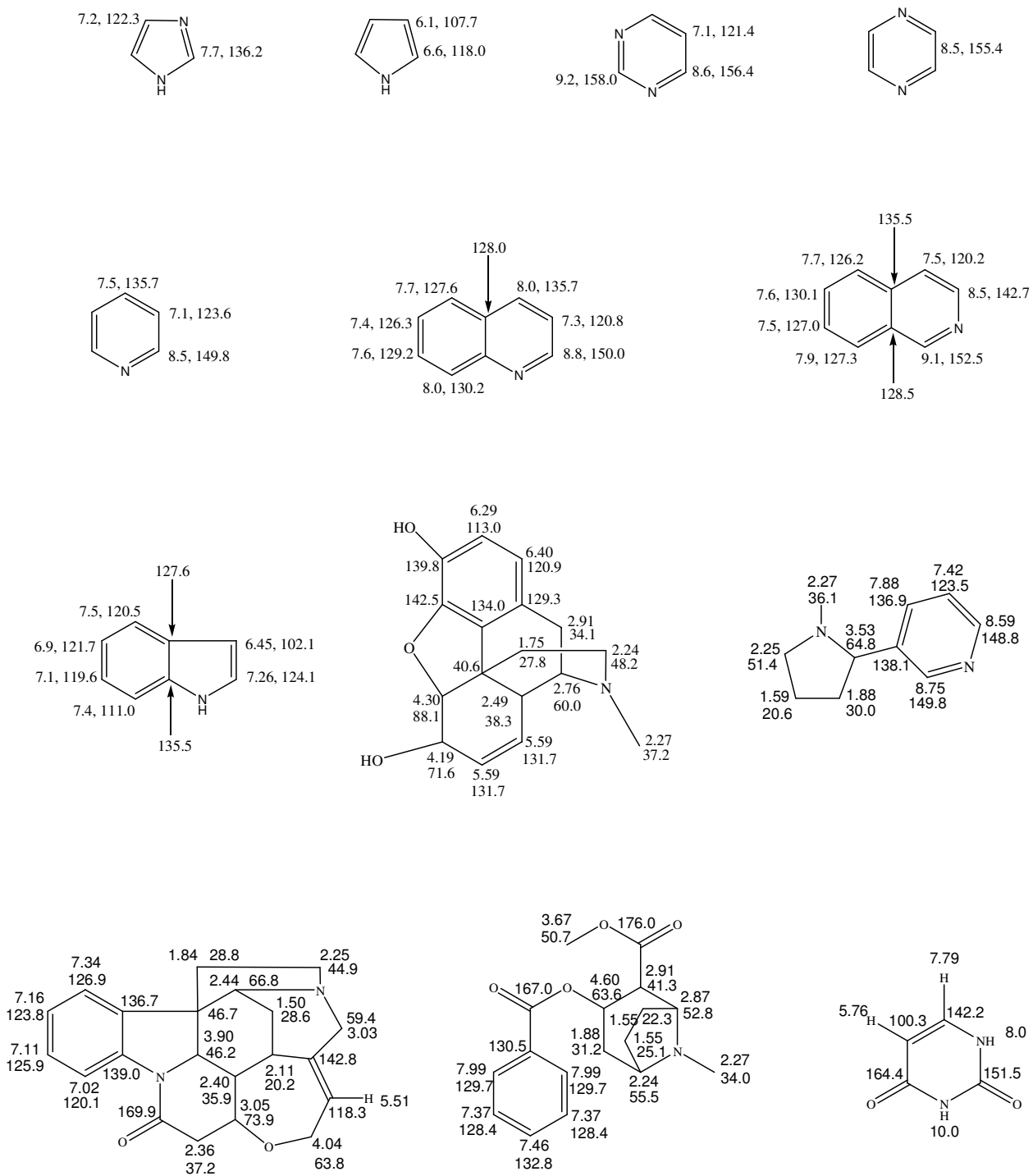


Fig. 13.  $^1\text{H}$  and  $^{13}\text{C}$  NMR values for some common nucleus

## One Dimensional NMR Spectroscopy

### <sup>1</sup>H NMR Spectroscopy

<sup>1</sup>H NMR technique gives the information about the position, nature and numbers of protons present in molecules. <sup>1</sup>H NMR spectrum can distinguish protons of different chemical environments. The spectrum shows direct proportionality between peak area and the number of nuclei responsible for the peak which is important for quantitative determination of any sample. This technique can also be employed for the determination of total concentration of a given kind of magnetic nuclei in a sample. It is widely used for determination of aspirin, phenacetin and caffeine for commercial analgesic preparations. In the structure determination of alkaloids, NMR spectrum shows clear appearance for aromatic hydroxyl ( $\delta$  8.0-10.5), aliphatic hydroxyl ( $\delta$  4.4-5.2), methoxyl ( $\delta$  3.5-4.3), N-methyl ( $\delta$  2.3-3.4), imine ( $\delta$  7.5-10.0), methylenedioxy ( $\delta$  5.8-6.2), aliphatic cyclic proton in close proximity to heteroatom ( $\delta$  2.5-3.2) and anomeric protons in case of alkaloid glycosides ( $\delta$  4.2-5.1) (Tanahashi et al., 2000; Kshiwaba et al., 2000).

### <sup>13</sup>C NMR Spectroscopy

<sup>13</sup>C nucleus is magnetically active like to <sup>1</sup>H nucleus having a spin number of ½. Since the natural abundance of <sup>13</sup>C is 1.1% to that of <sup>12</sup>C and its sensitivity is only about 1.6% to that of <sup>1</sup>H. Therefore this method is very sensitive for structure elucidation. <sup>13</sup>C NMR spectrum with its wide chemical shift range about  $\delta$  220 ppm (down field to TMS) has proved to be distinctly advantageous in structure elucidation of natural products (Wehrli and Wirthlin, 1976; Wehrli et al., 1989). Different alkaloids (Ramirez et al., 2003; Chen et al., 1997; Crabb, 1978; Levy et al., 1980) furnished characteristic peaks in <sup>13</sup>C NMR spectrum for carbonyl ( $\delta$  162-178), methoxyl ( $\delta$  51.5-58.2), oxygenation substituted aromatic carbon ( $\delta$  145-160), methylenedioxy ( $\delta$  98-104) and N-methyl ( $\delta$  25-45).

### Distortionless Enhancement by Polarization Transfer (DEPT)

This method is most efficient for the peaks assignments, whereby the peaks can be classified as representing methyl (CH<sub>3</sub>), methylene (CH<sub>2</sub>), methine (CH) or quaternary carbon (C). The novel feature in the DEPT sequence is a variable proton pulse that is set at 45<sup>0</sup>, 90<sup>0</sup> or 135<sup>0</sup> in three separate experiments. The signal intensity at a particular time for each of the three different pulses depends on the number of protons attached to a particular carbon atom. A separate sub-spectrum can

be recorded for each of the CH<sub>3</sub>, CH<sub>2</sub> and CH groups. In the DEPT spectrum, the multiplet components are not distorted, therefore leading itself to decoupling. This technique is therefore provides an alternative means for multiplicity determination (Doddrell et al., 1982; Bendall et al., 1982; Bendall et al., 1981). DEPT is usually being the method of choice when polarization transfer is required.

### **Insensitive Nuclei Enhanced by Polarization Transfer (INEPT)**

1D INEPT technique has proved to be a powerful tool for unambiguous carbon spectral assignment and also provides the information about critical carbon framework. Morris and Freeman, (1979); Cordell, (1991) used Selective INEPT spectroscopy (2D) for the multiplicity selection, spectral assignment and structure elucidation in some alkaloids. Bolcskei et al., (1994) applied the INEPT for structure elucidation of biologically active alkaloid, Catharanthine along with other 2D NMR technique. INEPT spectral analysis was used for determination of multiplicity in all carbon signals of dihydrochelerythrine, (±)-evodiamine and zanthobungeanine alkaloids (Joshi et al., 1991) for structure elucidation.

## **Two Dimensional NMR Spectroscopy**

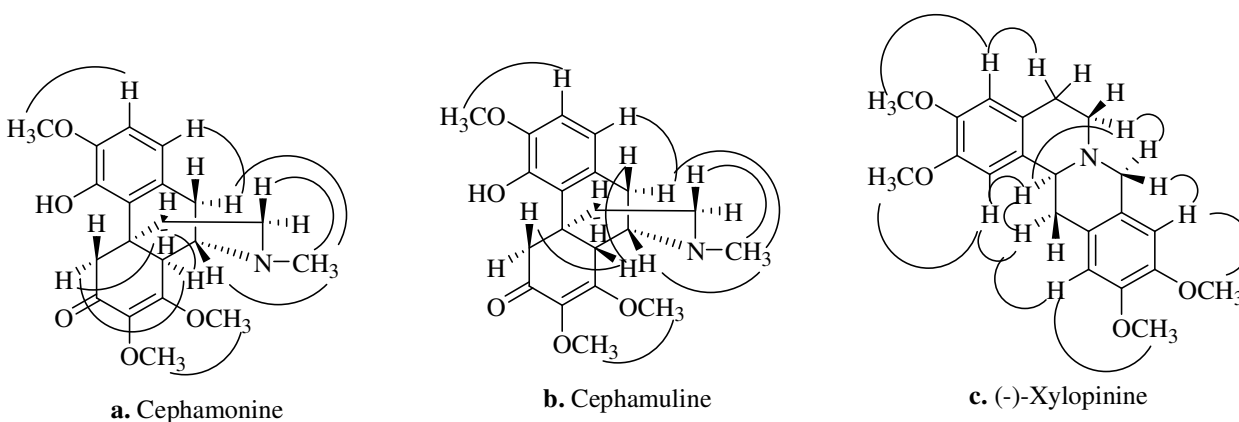
### **<sup>1</sup>H-<sup>1</sup>H Correlation Spectroscopy (COSY)**

2D NMR spectra provide more information about the chemical compounds particularly that are too complicated to work with using one-dimensional NMR. COSY was first discovery in the field of 2D experiments. It was proposed by Jean Jeener, Professor at Université Libre de Bruxelles, in 1971. This experiment was later implemented by Walter P. Aue, Enrico Bartholdi and Richard R. Ernst, who published their work in 1976 (Martin and Zekter, 1988). A 2D experiment which indicates all the spin-spin coupled protons in one spectrum is called <sup>1</sup>H-<sup>1</sup>H Correlation or COSY spectrum. In the general case, COSY spectra are interpreted as giving rise to off-diagonal or cross peaks for all protons that have significant *J*-*J* coupling. The cross peaks correlate coupled protons. In other words the experiment can be thought of as simultaneously running all pertinent decoupling experiments to see which protons are coupled to which other protons. In this method two identical chemical shift axes are plotted orthogonally. All peaks that are mutually spin-spin coupled detected as cross peaks and symmetrically placed about the diagonal. Csupor et al., (2004) established the structure of acovulparine a norditerpene alkaloid by using <sup>1</sup>H-<sup>1</sup>H-COSY experiment. In (+)-

sebiferine (Fig. 18a) (Bartley et al., 1994), an aliphatic ABC coupling system was evident involving the methylene hydrogens at  $\delta$  3.08, 3.38 and the methine hydrogen at  $\delta$  3.73; it was confirmed by observation of the expected cross-peaks in the COSY spectrum. Hughes et al., (1989) elucidated the structure of two lycopodium alkaloids, annotinine and annotine with the help of COSY experiments, geminal and vicinal coupling between protons in their structures was also discussed.

### Nuclear Overhauser Effect (NOE)

The interaction of one magnetic nucleus with another leading to spin-spin coupling takes place through the bounds of the molecules. NOE is useful for detection of groups which are close in space to each other and can provide the information about stereochemistry (Noggle and Schirmer, 1972) of molecule. The stereochemistry of cephamonine (Fig. 14a) and cephamuline (Fig. 14b) for H-14 was confirmed by NOE correlation and observed that in previous case H is  $\alpha$ -oriented while in later case it is  $\beta$ -oriented (Kashiwaba et al., 1994). The  $\alpha$ -,  $\beta$ -orientation of hydrogen in (-)-xylopinine (Fig. 14c) (Patra et al., 1987) was also confirmed by this technique.



**Fig. 14.** NOE correlations

### Nuclear Overhauser Enhancement Spectroscopy (NOESY)

A 2D spectrum which records all the  $^1\text{H}$ - $^1\text{H}$  NOE occurring in a molecule in a single experiment is called NOESY spectrum. Superficially, it appears like a  $^1\text{H}$ - $^1\text{H}$  COSY spectrum. NOESY spectrum provide crucial information about the geometry of the molecules. Joshi et al., (1994) established the structure of septatisine, a diterpenoid alkaloid, isolated from the roots of *Aconitum septentrionale* with the help of NOESY experiments. The actual configuration ( $\alpha$ -orientation) of OH group in isprostephabyssine (Fig. 15b) (Zhang and You, 2005) has been

significantly determined by NOESY spectrum. This compound is stereoisomer of prostephabyssine (Fig. 15a) that is different only in the orientation of OH group ( $\beta$ -orientation). These compounds verified the configuration of H-10, correlated with the N-methyl in prostephabyssine while no such correlation was observed in another case because of  $\alpha$ -orientation of OH group. The structure of azaanthracene alkaloid (Fig. 15c) (Roumy et al., 2006) was confirmed with the help of NOESY spectrum which showed all  $^1\text{H}$ - $^1\text{H}$  correlation present in its structure.

### Heteronuclear Overhauser Enhancement Spectroscopy (HOESY)

This is an important method utilized for structure elucidation and conformational analysis of small organic molecules (Yu and Levy, 1984; Rinaldi, 1983) as well as large bioactive compounds such as alkaloids and other natural products (Niccolai et al., 1985; Niccolai et al., 1984). In order to ensure that the magnetization transfer occurs via a dipolar mechanism, the HOESY pulse sequence effectively eliminates scalar coupling between  $^1\text{H}$  and  $^{13}\text{C}$  spins. A study of dipolar interactions between quaternary carbons and protons was carried out significantly for several natural compounds such as camphor (Yu and Levy, 1984), rifamycin S (Niccolai et al., 1985; Niccolai et al., 1984) and valinomycin (Khaled and Watkins, 1983) with the help of 2D HOESY.

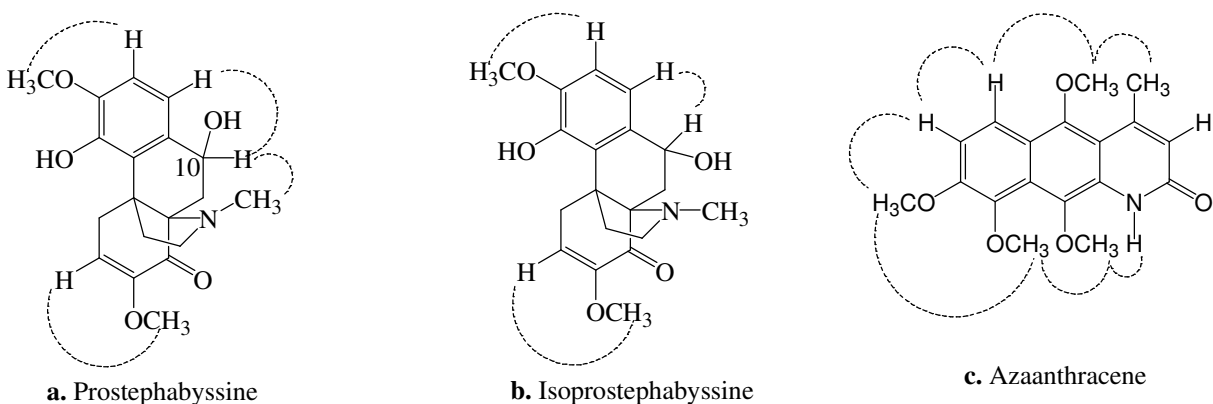


Fig. 15. NOESY correlations



### Heteronuclear Correlation Spectroscopy (HETCOR)

HETCOR or  $^1\text{H}$ - $^{13}\text{C}$  COSY experiment correlates  $^{13}\text{C}$  nuclei with differently coupled protons which is also known as one-bond ( $^1J_{\text{CH}}$ ) coupling (Derome, 1988; Williams and Fleming 2004). Joshi et al., (1997) elucidated the structures of various partially demethylated products from some aconitine-type norditerpenoid alkaloids named aconitine, cammaconine, delphinine, falconerine, lappaconitine, and talatizamine on the basis of HETCOR experiments. Demethylation process was carried out with trimethylsilyl iodide and HBr in glacial acetic acid.

### Incredible Natural Abundance Double Quantum Transfer Experiment (INADEQUATE)

This is useful technique to show direct  $^{13}\text{C}$ - $^{13}\text{C}$  correlations or connections in a compound through a 2D spectrum. This technique is highly sensitive since the natural abundance of  $^{13}\text{C}$  is only 1%, the probability of finding one  $^{13}\text{C}$  attached to another is only 1 in  $10^4$ . This technique is finds applications only where either extremely concentrated solution of sample or the molecule is enriched in the  $^{13}\text{C}$  isotope.

### Abundance Double Quantum Transfer Experiment (ADEQUATE)

This technique is used for the determination of direct correlations between  $^1\text{H}$  and  $^{13}\text{C}$  nuclei in an organic compound. The structure of azaanthracene alkaloid (Fig. 16) (Roumy et al., 2006) was confirmed with the help of ADEQUATE spectrum which showed all  $^1\text{H}$ - $^{13}\text{C}$  correlation present in its structure.

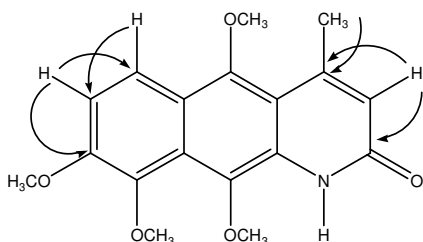


Fig. 16. ADEQUATE correlations in azaanthracene

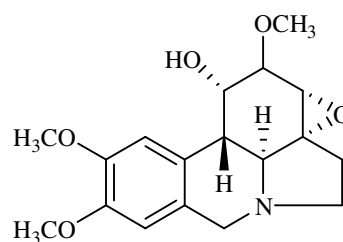


Fig. 17. Incartine

### **Heteronuclear Single Quantum Coherence (HSQC)**

HSQC experiments showed  $^1\text{H}$ - $^{13}\text{C}$  correlation using pulse sequence in spinning nuclei. The structure of four alkaloids 16,17-dihydro-17 $\beta$ -hydroxy isomitraphylline; 16,17-dihydro-17 $\beta$ -hydroxy mitraphylline; isomitraphylline and mitraphylline (Pandey et al., 2006) were elucidated with the help of HSQC along with other 2D techniques. The structure and stereochemistry of lymphostin alkaloid (Aotani et al., 1997) was determined by this method and elucidated as pyrrolo[4,3,2-de]quinoline.

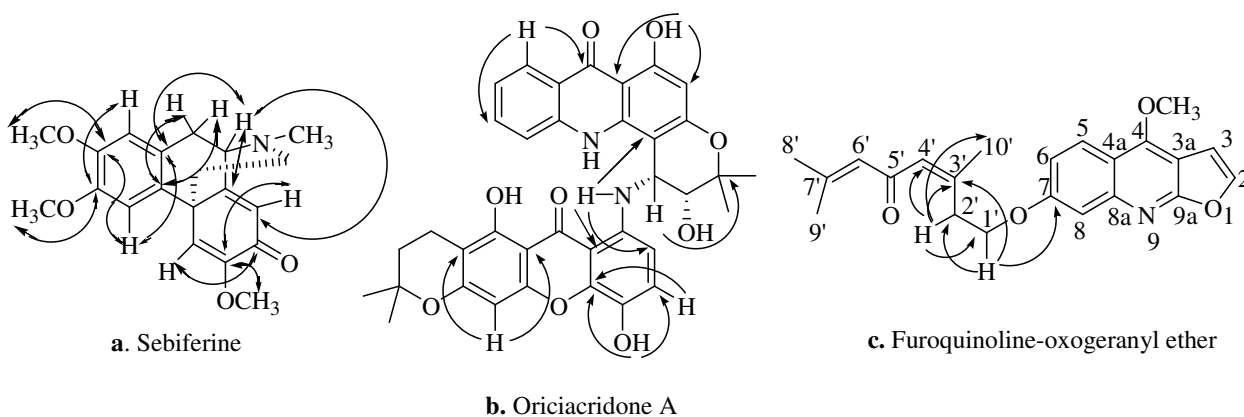
### **Heteronuclear Multiple Quantum Coherence (HMQC)**

In this technique the correlation is achieved between  $^{13}\text{C}$  and  $^1\text{H}$  (Martin and Zekter, 1988). Zellagui et al., (2004) have successfully elucidated the structures of lupanine and S-calycotomine by using HMQC experiments. The 1H-1H-COSY, HMQC and HMBC experiments established geminal and vicinal hydrogen interactions as well as direct ( $^1J_{\text{CH}}$ ) and two and three bond correlations between carbon and hydrogen atoms in the structure of a natural quaternary alkaloid, N-methylvoachalotine (Medeirosa et al., 2001). HMQC experiments of incartine alkaloid (Fig. 17) isolated from *Galanthus elwesii* (Berkov et al., 2007) showed clear multiplicity for each carbons and correlation of these carbons to their respective proton. In its spectrum, three quartate signals at  $\delta$  59.2, 56.1, 56.2 for the methoxy groups, three triplets at 56.7, 30.1 and 53.0 corresponding to the methylene carbons, two doublets at 110.7 and 107.6 assigned to the aromatic carbons, five singlets at 66.9, 128.5, 147.8, 147.9 and 127.9 assigned to the quaternary carbons and five doublets at 67.7, 78.2, 59.0, 61.3, and 40.1 corresponding to the remaining carbons in the structure.

### **Heteronuclear Multiple Bond Correlation (HMBC)**

HMBC is also known as long-range  $^1\text{H}$ - $^{13}\text{C}$  COSY in which a time delay in the pulse sequence is set to correspond to  $^{1/2}J$  where  $J$  is in the region of 10 Hz. Since many two bond and three bond coupling constant are rather similar in value and lie in the range of 2-20 Hz, then  $^{13}\text{C}$  chemical shifts correlated with the chemical shifts of those protons separated from them by two and three bonds. The relative positions of the methoxy substituents and the isolated unsaturated hydrogens in the structure of sebiferine (Fig. 18a) were confirmed by multiple bond correlation spectroscopy using the HMBC sequence (Bartley et al., 1994). Wansi et al., (2006) furnished the exact structure of oriciacridone A (Fig. 18b) by using long-range  $^1\text{H}$ - $^{13}\text{C}$  correlation. Chemical structure and the relative stereochemistry of alkylated piperazine alkaloid named 3,5-diisobutryl-6-isopropyl-piperazine-2-one (El-Desouky et al., 2007) was elucidated on the basis of HMBC

technique. The unambiguous structure of the side-chain in furoquinoline oxogeranyl ether (Fig. 18C) (Inada et al., 2008) was established by HMBC experiments to indicate significant correlation peaks between H-1' / C-2', C-3', C-7; between H-2' / C-1', C-3', C-4', C-10'; between H-4' / C-2', C-5', C-10' and between H-6' / C-5', C-8', C-9'. The connection of the 5-oxogeranyl side-chain to C-7 on the furoquinoline ring was revealed by the HMBC correlation peak between H-1' / C-7. Li et al., (2001) were noted the usefulness of the gradient  $^1\text{H}$ - $^{15}\text{N}$  HMBC NMR spectroscopy in the structure elucidation of 3-hydroxyrutaecarpine.



**Fig. 18.** HMBC correlations

## Mass Spectrometry

The mass spectrometry is an essential tool for identification of organic compound. It provides accurate molecular weights, elemental information and fragmentation pattern, correct interpretation of which helps to determine the structure of unknown compound. The structure elucidation of alkaloids by mass fragmentation pattern (Biemann, 1962; Budzikiewicz et al., 1964) is easier than the other natural products because of complexity in their structure. Qian and Zhan, (2007) elucidated the structures of various alkaloids such as sessilifolines A, B, tuberstemonine, sessilifoliamide A and stemoninoamide, isolated from the stems of *Stemona sessilifolia* with the help of simple mass fragmentation pattern. Various techniques used under mass spectrometry for structure elucidation are as follows;

## Electron Impact Mass Spectrometry (EIMS)

The volatile compounds introduced by vaporization or by GC through a very small orifice followed by ionization by electron impact, determined under this procedure. The structures of acovulparine (Csupor et al., 2004) a norditerpene alkaloid was established by HR-EIMS. EIMS spectrum of parvineostemonine (Ke et al., 2003; Pilli and Oliveria, 2000) suggest that it gave molecular ion peak at  $m/z$  275, the other intense peaks were assign at 246 (Loss of ethyl group from  $M^+$ ) and 137 (perhydroazaazulene skeleton). Jones et al., (1999) proposed the fragmentation for pyridine alkaloid 2-[3-(6-propyl-2-pyridyl)-propyl]-1,3-dioxolane (Fig. 19). Santos et al., (2003) described the mass fragmentation in lignoaporphine alkaloid (Fig. 20) using EIMS method at 70 eV.

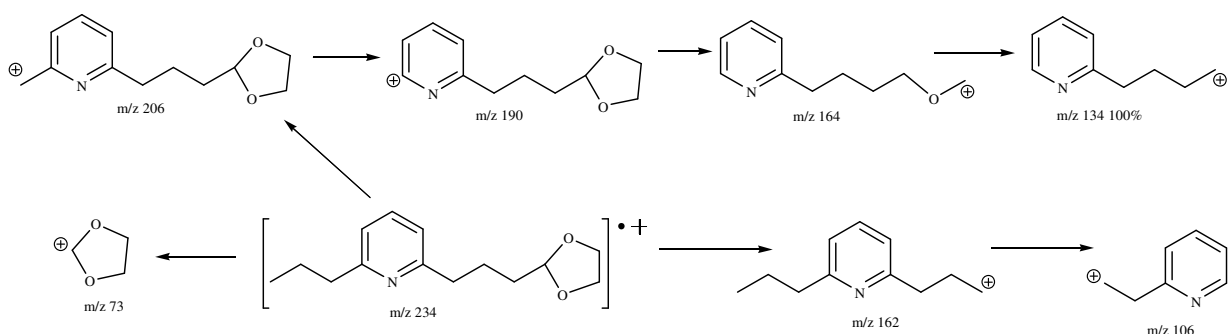


Fig. 19. Mass fragmentation in pyridine alkaloids

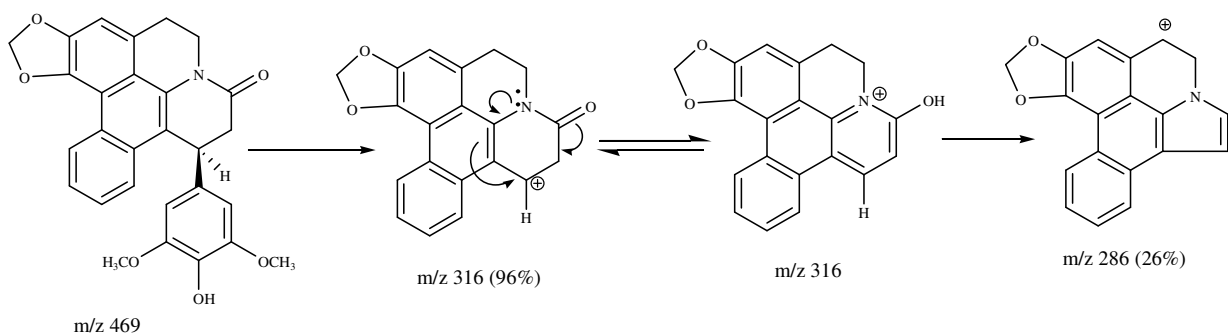
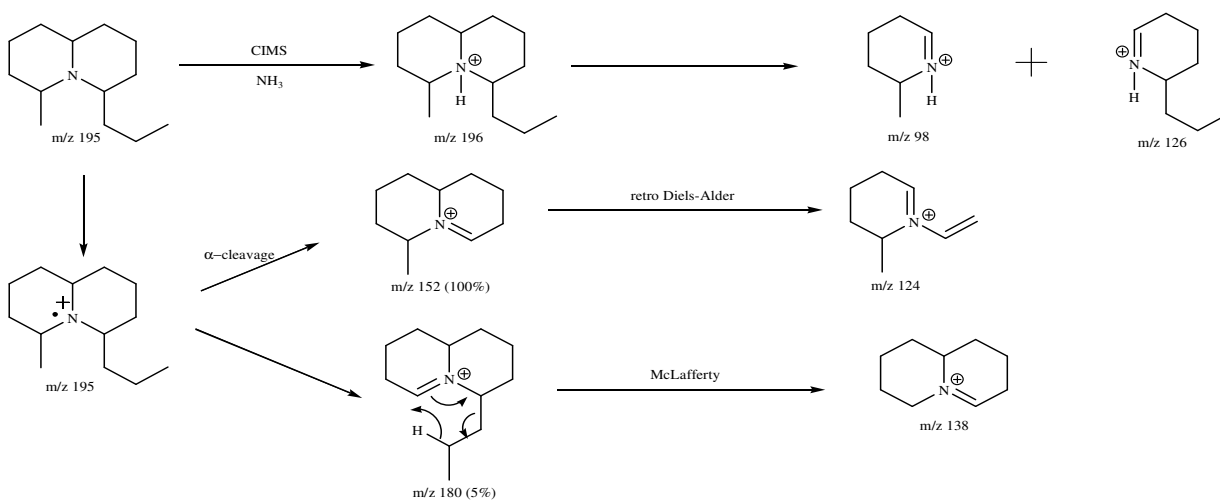


Fig. 20. Mass fragmentation of lignoaporphine alkaloid

## Chemical Ionization Mass Spectrometry (CIMS)

The vaporized sample is introduced into the mass spectrometer with an excess of a reagent gas (commonly methane) at a pressure of about 1 torr. In these spectral experiment the  $[M+H]^+$  ions (quasimolecular ions) are often prominent (Silverstein and Webster, 1998) Mass spectra of several *Erythrina* alkaloids (Boar and Widdowson, 1970) and their derivatives, arundamine and arundanine (Khuzhaev, 2004) were recorded by using CIMS method. Jones et al., (1999) determined the

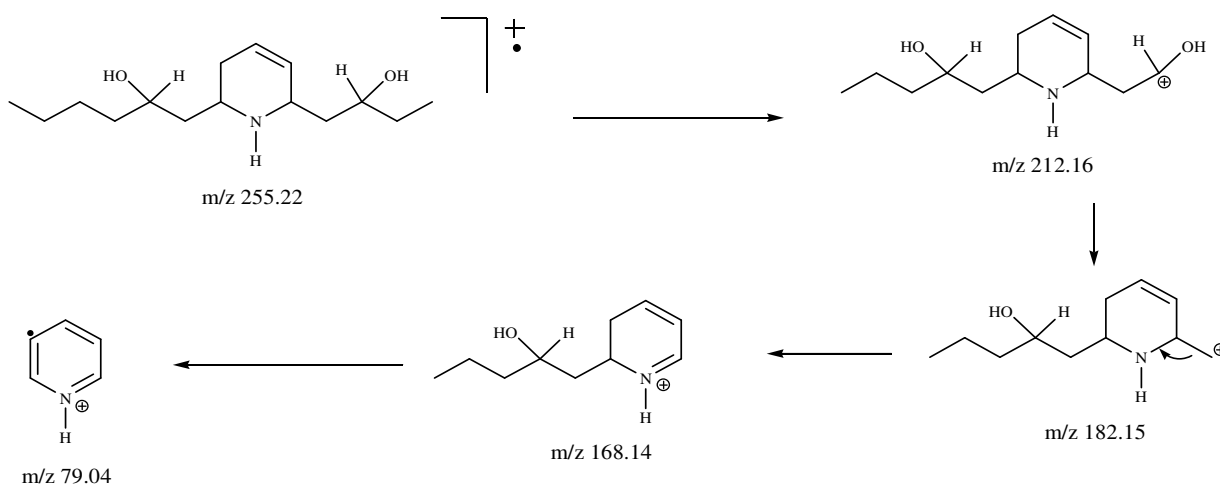
structure and chemistry of several quinoline and dihydroquinoline of animal origin and proposed a mechanism of mass fragmentations in quinolizidine (Fig. 21).



**Fig. 21.** Mass fragmentations of quinolizidine

### Field Desorption Mass Spectrometry (FDMS)

This method is used for non or low volatile and thermally labile substances. Usually the major peak represents the  $[M+H]^+$  ion. For molecular weight determination of large and polar substances, a small amount of cation donor reagents (Prome and Puzo, 1977) is added to the sample. Some times due to inorganic impurities in sample produce most intense  $[M+Na]^+$  and  $[M+K]^+$  peaks. Ahmad et al., (2002), reported the fragmentation pattern in piperidine alkaloids isolated from *Andrachne aspera* with the help of EI/FD/HRMS experiments. They proposed a suitable rout for fragmentations in aspertin B is shown in Fig. 22.



**Fig. 22.** Fragment ions in FDMS/HRMS of aspertin B

## Fast Atomic Bombardment Mass Spectrometry (FABMS)

Polar molecules such as peptide and several others with molecular weights upto 10,000 Da can be analyzed by this soft ionization technique. The bombarding beam consists of xenon or argon atoms of high translational energy. This beam is produced by first ionizing xenon atoms with electrons to give xenon radical cations. The sample compound is dissolved in a high boiling viscous solvent such as glycerol, a drop is placed on a thin metal sheet and the compound is ionized by the high energy beam of xenon atoms. Although the molecular ion itself is usually not seen but adduct ions such as  $[M+H]^+$  are prominent. Other adduct ions can be formed from salt impurities or upon addition of salts such as NaCl or KCl, which give  $[M+Na]^+$  and  $[M+K]^+$  additions. FABMS requires a more simple instrumentation (Fenselan, 1978) which is easy to operate and has been used more frequently. Belferdi and Belattar, (2001) suggested a suitable route for mass fragmentation pattern in oxoaspidospermidine-N-oxide (Fig. 23) in which all the rearrangements in the molecule are shown.

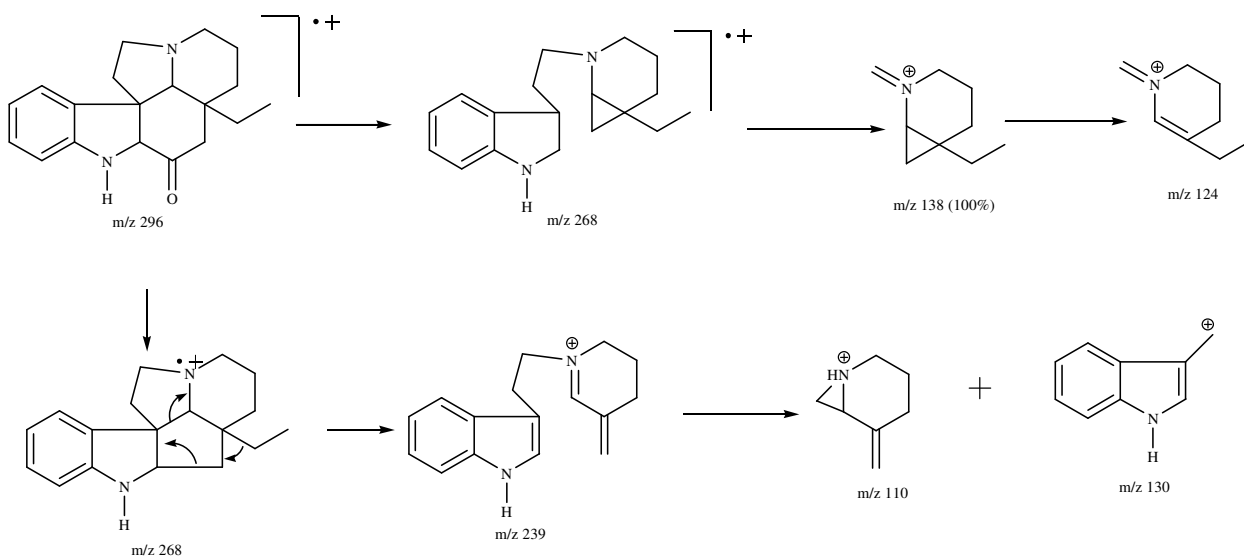


Fig. 23. Mass fragmentation ions of oxoaspidospermidine-N-oxide

## Electrospray Ionization Mass Spectrometry (ESIMS)

This method involves placing an ionizing voltage of several kilovolts across the nebulizer needle attached to the outlet from a HPLC. This technique is widely used on water soluble molecules such as carbohydrates, protein and peptide. Ergot alkaloids (Lehner et al., 2004) ergovaline (m/z 534), ergotamine (m/z 582), ergocornine (m/z 562), ergocryptine (m/z 576) and ergocrystine (m/z 610) exhibited a consistent loss of water (-18 u) from the C-12'  $\alpha$ -hydroxy functionality. Of this group, ergovaline and ergotamine generated an m/z 320 fragment deriving from cleavage of ring E

amide and ether functions with retention of the peptide ring system methyl group. Ergocornine, ergocryptine and ergocrystine similarly formed an  $m/z$  348 fragment with retention of isopropyl. Fragmentations of aporphine alkaloids (Caroline et al., 2004) by electrospray ionization with multistage mass spectrometry (ESI-MS<sup>n</sup>) in positive mode showed the loss of the amino group and its substituents in first step. Further steps display the loss of the peripheral groups, losses of methanol and CO are observed if an OH is vicinal to an OCH<sub>3</sub> on the aromatic ring. Otherwise the spectra show radical losses of CH<sub>3</sub><sup>·</sup> or CH<sub>3</sub>O<sup>·</sup> as the main fragmentations. If a methylenedioxy group is present losses of formaldehyde followed by CO are observed. These fragmentations provide important information on the structures of aporphine alkaloids. A typical ESI spectrum of aconitine alkaloid (Sun et al., 2004) showed protonated molecular [M+H]<sup>+</sup> ion and major fragment ion by the loss of neutral molecular species of  $m/z$  60 (CH<sub>3</sub>COOH), 122 (PhCOOH), 152, 279 etc.

### **Matrix Assisted Laser Desorption Mass Spectrometry (MALDIMS)**

This method has been used recently in several variations to determine the molecular weight of large molecules upto several hundreds kDa. The successful use of six beta-carbolines, harmaline, harmine, harmol, harmaline and harmalol (Nonami et al., 1997) is reported as matrices in matrix-assisted MALDI/TOF-MS ( $\lambda_{\text{max}}$  337 nm) for proteins and sulfated oligosaccharides, using stainless-steel probes and membranes to prepare the samples. The usefulness of the new matrices in MALDI/TOF-MS of proteins and sulfated oligosaccharides is compared with those of classical MALDI matrices such as alpha-cyano-4-hydroxycinnamic acid, gentisic acid, sinapinic acid, 6-aza-2-thiothymine and 3-indole- trans-beta-acrylic acid. Direct quantitative analysis using TLC coupled with MALDIMS has been demonstrated, an internal standard and a data collection protocol were used for analysis directly from TLC plates to compensate for shot-to-shot signal degradation, as well as deviations of analyte and internal standard spatial distributions within the TLC spot. Cocaine hydrochloride (Nicola et al., 1996) was used as a model compound for this study, and cocaine itself used as the internal standard.

## Gas Chromatography-Mass Spectrometry (GCMS)

Gas chromatography coupled with mass spectrometry (GC-MS) has been used successfully for the identification of tropane alkaloids (Christen et al., 1993). The capillary gas chromatography with mass spectrometry (GC/MS) is convenient and very often used method for analysis of a complex mixture containing pyrrolizidine alkaloids (Witte et al., 1993). GCMS spectrum of 3- $\alpha$ -cinnamoyloxypiperydine (Fig. 24) (Munoz and Casaleb, 2003) having molecular formula  $C_{17}H_{21}NO_2$  exhibited the fragmentation pattern similar to 3-substituted tropane (Christen et al., 1995). The molecular ion peak at  $m/z$  271, a base peak at  $m/z$  124  $[M - PhCH=CHCOOH]^+$  along with other strong peaks at  $m/z$  148  $[PhCH=CHCOOH]^+$ , 140  $[M - PhCH=CHCO]^+$ , 131  $[PhCH=CHCO]^+$ , 103  $[PhCH=CH]^+$ , and 77  $[C_6H_5]^+$ . The similar fragmentation was found in 3-(3'-formyloxypiperydineoxy) tropane (Berkov, 2003) which showed molecular ion at  $m/z$  317 ( $C_{18}H_{23}NO_4$ ) and most abundant ion at  $m/z$  124 (tropane group) together with ion at  $m/z$  140 ( $M^+ - 177$ ), 103. The chemical profiling of alkaloids from Bulgarian species of genus *Senecio* (Christova and Evstatievab, 2003) was successfully carried out by the GC/MS analysis.

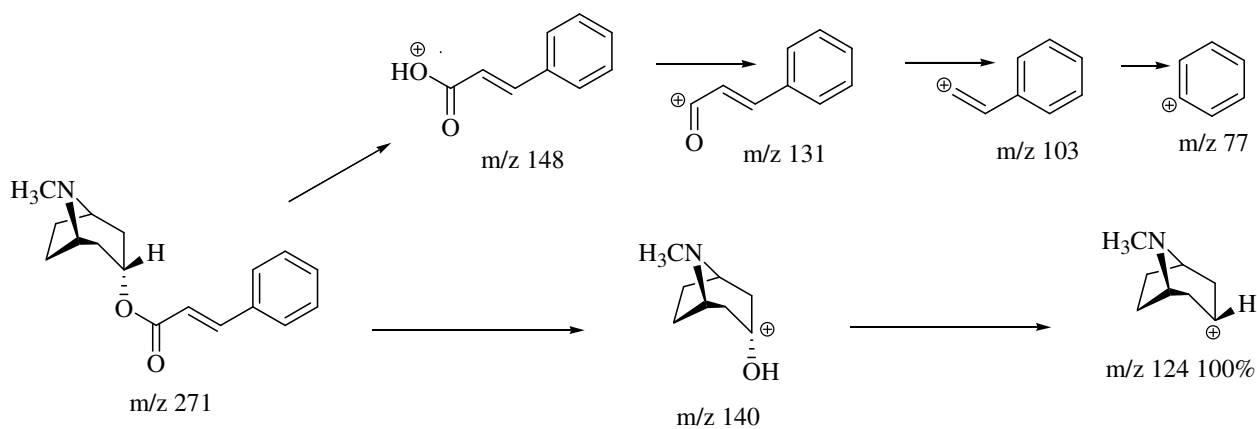


Fig. 24. Mass fragments of 3- $\alpha$ -cinnamoyloxypiperydine in GCMS

## Liquid Chromatography-Mass Spectrometry (LCMS)

LCMS of any component facilitate quick characterization. The sample is dissolve in a suitable solvent and separated its components with the help of LC and finally determined the molecular weight and mass fragmentation in a particular component. Solid-phase extraction (cation-exchange) of pyrrolizidine alkaloids and their N-oxides from honey samples (Kerrie et al., 2004; Betteridge et al., 2005) was carried out by high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. Enantio-enriched building blocks (Feng et al.,



2006), several ephedrine type alkaloid (Gay et al., 2001; Thomas et al., 2007; Sharpless et al., 2006; Joshi and Khan, 2005; Gray et al., 2004; Trujillo and Sorenson, 2003) were determined with the help of LCMS using atmospheric pressure chemical ionization. Aconitum alkaloid (Hajime et al., 2001) was analysed significantly from aconite roots with the help of LCMS technique. A semi-quantitative LC-MS method (Beike et al., 2003) was developed for the detection of the pseudo alkaloids of *Taxus baccata*. This method was used to examine the cause of death of a 43 year old man who died several hours after he drank a decoction of taxus leaves.

### **X-Ray Diffraction**

Many compounds are capable of crystallizing in more than one type of crystal lattice. At a particular temperature and pressure, only one crystalline form is thermodynamically stable. It is used to find several polymorphs of crystalline alkaloids existing under normal handling conditions. Solubility, melting point, density, hardness, crystal shape, optical and electrical properties, vapour pressure, etc. vary with polymorphic forms. Powder X-ray diffraction analysis is employed for the characterization of crystalline structure. It has been used to determine the existence of polymorphic forms of many substances such as carbon in graphite or diamond and drugs. Various other techniques such as diffraction topography, double crystal diffractometry, interferometry, strain measurements and texture analysis are very important techniques used for the study of crystal perfection, structure defect, grain structure and orientation. The structure of altaconitine alkaloid (Batbayar et al., 1993) isolated from the epigeal part of *Aconitum altaicum* was established on the basis of x-ray structural method along with IR,  $^1\text{H}$  NMR,  $^{13}\text{C}$  NMR and MS studies.

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