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Natural aphrodisiacs. Studies of commercially-available herbal recipes, and phytochemical investigation of *Erythroxylum vacciniifolium* Mart. (Erythroxylaceae) from Brazil

Thèse de doctorat

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A mes parents et ma sœur

A Laura

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Publications

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Posters

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Abbreviations

$[\alpha]_D$	specific rotation at the wavelength D of sodium
Abs.	absorbance
ACh	acetylcholine
AChE	acetylcholinesterase
ACTH	adrenocorticotrophic hormone
APCI	atmospheric pressure chemical ionization (MS)
APG	Angiosperm Phylogeny Group
API	atmospheric pressure ionization
br s	broad singulet
cAMP	cyclic adenosine monophosphate
CC	column chromatography
CD	circular dichroism
cGMP	cyclic guanosine monophosphate
CI	confidence interval
CNS	central nervous system
Col2	colon cell lines
d	doublet (NMR)
D/CI	desorption/chemical ionization (MS)
Da	Dalton
DCM	dichloromethane
δ	chemical shift (ppm) (NMR)
dd	double doublet (NMR)
DEPT	distortionless enhancement by polarisation transfer (NMR)
DHEA	dehydroepiandrosterone
DMSO	dimethylsulfoxide
EI	electron impact (MS)
ESI	electrospray ionization (MS)
Et_3N	triethylamine
EtOH	ethanol
FIA	flow injection analysis
GABA	γ-aminobutyric acid
gDQF-COSY	gradient double quantum filtered correlation spectroscopy (NMR)
gHMBC	gradient heteronuclear multiple bond correlation (NMR)
gHSQC	gradient heteronuclear single quantum coherence (NMR)
Hdmb	4-hydroxy-3,5-dimethoxybenzoic acid
HPLC	high performance liquid chromatography
HR	high resolution (MS)
HRESMS	high-resolution electrospray ion cyclotron resonance mass spectrometry
hTERT-RPE1	human telomerase reverse transcriptase-retinal pigment epithelial cells

HUVEC	human umbilical vein endothelial cells
Hz	Herz
i.d.	internal diameter
IR	infrared spectrum
J	coupling constant (NMR)
KB	oral epidermoid cell lines
LC-DAD-UV	high performance liquid chromatography coupled with ultraviolet photodiode array detector
LC-MS	high performance liquid chromatography coupled with mass spectrometry
LC-NMR	high performance liquid chromatography coupled with nuclear magnetic resonance spectroscopy
LNCaP	hormone-dependant prostate cell lines
Lu1	human lung cancer cell lines
m	multiplet (NMR)
MAO	monoamine oxidase
$[M+H]^+$	protonated molecule
$[M_D+D]^+$	deutered molecule
m/z	mass per electronic charge
MDR	multidrug-resistance
MeCN	acetonitrile
MeNE	methylnorepinephrine
MeOH	methanol
Mpc	1-methyl-1*H*-pyrrole-2-carboxylic acid
MPLC	medium pressure liquid chromatography
MS	mass spectrometry
MS^n	multiple stage tandem MS
MW	molecular weight
NE	norepinephrine
NMR	nuclear magnetic resonance
NO	nitric oxide
NOE	nuclear overhauser effect (NMR)
NOESY	nuclear overhauser effect spectroscopy (NMR)
NOS	nitric oxide synthase
PDE	phosphodiesterase
ppm	parts per million (NMR unity)
Pc	1*H*-pyrrole-2-carboxylic acid
RSD	relative standard deviation
RT	retention time
R.T.	room temperature
s	singlet (NMR)
SD	standard deviation
sp.	unspecified species (one)

SP-HPLC	semi-preparative high performance liquid chromatography
spp.	species (several)
ssp.	subspecies
t	triplet (NMR)
TLC	thin-layer chromatography
TMS	tetramethylsilane
TOCSY	total correlation spectroscopy (NMR)
TOF	time of flight (MS)
UV	ultraviolet spectrum
VLB	vinblastine
WET	fast solvent suppression (LC-NMR)
yohimbine-d_3	deuterium-labeled yohimbine

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I. Aim of the work

Throughout all ages, men and women have incessantly pursued every means to increase, preserve and recapture their sexual capacity, or to stimulate the sexual desire of selected members of the opposite or same sex. One of the most recurrent methods has been the use of aphrodisiacs. Herbal medicines are a major source of aphrodisiacs and have been used world-wide for thousands of years by different cultures and civilizations. Traditional herbs represent an extraordinary reservoir of active ingredients which are still present in about 25% of all prescriptions of modern "Western" medicine. Phytochemicals have evolved from traditional medicinal plants to modern scientific medicine, giving support to the empirical knowledge of "alternative" healing. A deeper understanding of phytochemistry, pharmacognosy and ethnopharmacology should therefore be encouraged to support the production of new and safe pharmacologically active compounds with minimal undesired toxic effects.

Nowadays, the commercialization of new synthetic "love drugs", *e.g.* Viagra®, Cialis® and Levitra®, has fascinated the public interest and has led to a reassessment of classical aphrodisiacs and to the search for new ones. The resurgence of "old" herbal remedies that arouse sexual activity and the use of new exotic preparations has coincided with an increase in poisonings associated with the use of these aphrodisiacs. The practice of self-medication by an increasing number of patients, the incessant aggressive advertising of these herbal aphrodisiacs, the invasion of the medicinal market with uncontrolled dietary supplements and the absence of real directives amplifies the potential health hazards to the community.

With the aim of evaluating the potential risks of commercialized aphrodisiac products on consumer health, the first part of the present work was to develop a new analytical method to detect and quantify yohimbine in these herbal remedies. Yohimbine is the active principle of *Pausinystalia yohimbe* PIERRE ex BEILLE (Rubiaceae), a classical aphrodisiac which has been recently revalued for its pro-sexual properties and extensively commercialized without any surveillance.

The second part of this work is focused on the phytochemical and pharmacological investigation of *Erythroxylum vacciniifolium* Mart. (Erythroxylaceae), a plant used in Brazilian traditional medicine as an aphrodisiac and tonic, and locally known as *catuaba*. The phytochemical and pharmacological aspects consist in the detection, isolation and characterization of new natural compounds of potential therapeutic interest, especially for the treatment of male erectile dysfunction.

II. Introduction

1. Aphrodisiacs: the eternal dream

Throughout all times and at all social and cultural levels, one of the leading forces that has driven humanity to triumphant achievements, exultant productivity, and personal satisfaction, is the amatory concept. The proverbial belief that the world is conditioned by love is not an unfounded saying. The idea of love as the instrument of creation and human perpetuity is not a merely mechanical conception. In the first century B.C., the ancient Roman poet Lucretius (99-55 B.C.) began his epic philosophical poem *De Rerum Natura* (*On the Nature of the Universe*) with a justly famous invocation to Venus, the poet's symbol for the forces of cohesion, integration and creative energy in the universe. The Lucretian Venus is associated with the figure of love, the unifying or binding energy, presented by the poet as a kind of life principle (Lucretius and Giussani, 1967). In the name of the goddess, terrible and wicked crimes have been committed, men have defied uncontrolled dangers, have renounced magnificence, power, and possessions, confirming the quasi virtue of "All for love".

Therefore, it is not surprising that man has incessantly pursued every means, every obscure aid, to maintain his amorous faculties. Erotic vigor, in all its varying manifestations, has become for man a prior interest, and woman the complement in this function. Thus we can consider that human history, from past to present, is run by the two harmonizing principles of creation and survival, male and female, permeating the creative arts, painting, sculpture, music, literature and simpler doings of ordinary life.

The eternal dream of man and woman have always been the possibility of increasing, preserving and recapturing their sexual capacity, or of stimulating the sexual desire of selected members of the opposite or same sex by various means. One of the most recurrent methods has been the use of aphrodisiacs. These products, causing or increasing sexual desire or arousing sexual response, may simply be exotic food or drinks, rare herbal compounds or pharmaceuticals, amulets or psychic manipulations. Even putatively innocuous foods can be used as aphrodisiacs by suggesting or resembling sex organs (asparagus, oysters, *etc.*). Human sexual arousal is a complicated phenomenon, but simple conditioning stimuli of different genres can act as potent aphrodisiacs.

From a purely biological point of view, maintaining or increasing amorous capabilities can have varying importance between the two sexes, due to their basic physiological inequality. In nature, the only requirement for a successful sexual encounter is that the male should be able to achieve penetration and ejaculation. But from a social point of view, both partners are looking for sexual pleasure and satisfaction. Women are then biologically disadvantaged in the achievement of the satisfaction, as we are designed so that the male climaxes before the female in order to propagate the species. The chance of getting pregnant would be by far reduced if

female was the first to reach sexual pleasure. On the other hand, women have the significant advantage of being able to achieve multiple orgasms and probably more pleasure than man. Different from other animals, the civilized man has the time and opportunity to treat sex as a pleasurable activity rather than as an essential act for the propagation of the species. However, it is not just for pleasure that man has always hunted for substances to increase his sexual activities.

Sexual potency is part of the male ego, and the anxiety and the humiliation that are associated with a declining sexual ability are common to all cultures. That is why the popularity of aphrodisiacs has not shown any sign of decline throughout history. In ancient as in current times, in oriental and western cultures, virility has always been one of the central and most desirous characteristics of the human male. During the course of centuries, the cult of the phallus has assumed divine proportions, as it is considered the symbol of creation and life. Its representation, as farce or transcendent service, is omnipresent in ancient and modern arts. Images of the Greek god Priapos (the protector of gardens and domestic animals and fruits, son of Dionysus and Aphrodite) with large ithyphallic genitals were placed in the fields and gardens to ensure fruitfulness and protection. He was imported into Rome from Lampascus where Pausanias reported that he was supreme among all gods. The Roman Priapus was far more popular than his Greek version. He is portrayed wearing a long dress that leaves the genitals uncovered (Figure 0-1). The Romans placed a satyr-like statue of him, painted red and with an enormous phallus, in gardens as some kind of scarecrow, but also to ensure fruitfulness.

Figure 0-1

Priapus, the Roman patron god of gardens, vinicultures, sailors and fishermen.

The celebration of virility and glorification of manhood have also been testified throughout all ages by other cultures and ethnic groups: in ancient Babylonia as well as in India (Caves of Ajanta), in the medieval period (Courts of Love) and in contemporary times, in Mayan and Incan civilizations as well as in Polynesian and Cameroonian tribes, and so forth.

Sexual virility still covers an important role in contemporary society, as shown by the omnipresence of insistent commercials and flourishing businesses based on this theme. The wide diffusion of a new means of communication at the end of last century, the Internet, has contributed to the extension of this kind of commerce all over the world in varying degrees. Aphrodisiacs of all varieties and from the most remote origins become accessible to everyone, increasing in some way the popular curiosity and demand for these "magic potions". Moreover, the arrival of new synthetic "love drugs", *e.g.* Viagra®, Cialis® and Levitra®, has captivated the public imagination and has led to a reassessment of "old" aphrodisiacs and to the search for new ones (Drewes *et al.*, 2003).

2. Definition

Aphrodisiacs take their name from Aphrodite ("*Αφροδιτη*"; etymology: "foam-born") (Figure 0-2), the Greek goddess of love, and have been used throughout all times with the purpose of increasing desire and drive associated with the sexual instinct. According to Hesiod, Aphrodite was born when Uranus (the father of the gods) was castrated by his son Cronus. Cronus threw the severed genitals into the ocean which began to churn and foam about them. From the *aphros* ("sea foam") arose Aphrodite, and the sea carried her to Cythera (Figure 0-3). Aphrodite loved and was loved by many gods and mortals. Among her mortal lovers, the most famous was perhaps Adonis. Some of her sons are Eros, Anteros, Hymenaios and Aeneas (with her Trojan lover Anchises). She is accompanied by the Graces. Her priestesses were not prostitutes but women who represented the goddess and sexual intercourse with them was considered just one of the methods of worship. Aphrodite was originally an old-Asian goddess, similar to the Mesopotamian Ishtar (the ancient Sumero-Babylonian goddess of love and fertility) and the Syro-Palestinian goddess Ashtart. To the Romans she was Venus, the irresistibly beautiful goddess of love. Venus is the daughter of Jupiter, and some of her lovers include Mars and Vulcan, modeled on the affairs of Aphrodite. Venus' importance rose, and that of her cult, through the influence of several Roman political leaders. The dictator Sulla made her his patroness, and both Julius Caesar and the emperor Augustus named her the ancestor of their family (Oswalt, 1969).

Figure 0-2

Aphrodite, known as the Venus de Milo (Venus of Melos), Greece, second century B.C. (Marble statue, Louvre, Paris).

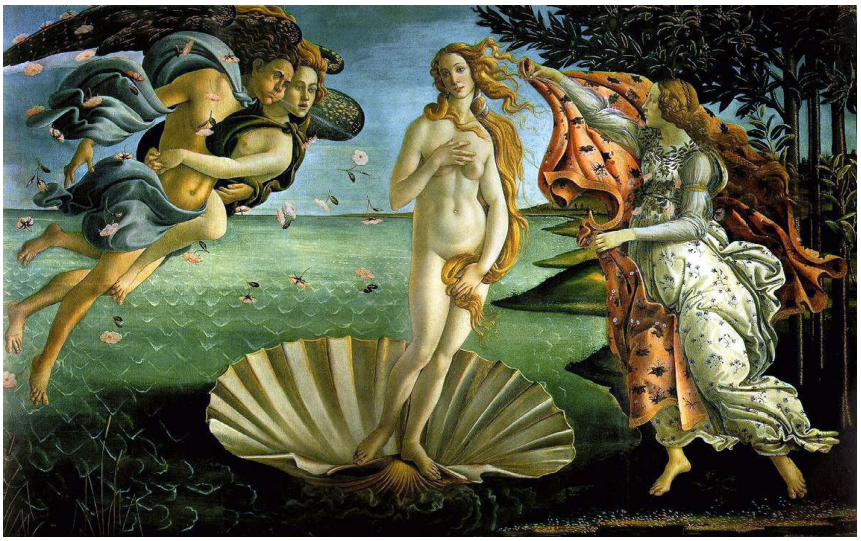


Figure 0-3

"The birth of Venus", Sandro Botticelli *ca.* 1482 (Tempera on canvas, Uffizi, Florence).

The *Oxford English Dictionary* defines an aphrodisiac as "A drug or preparation inducing venereal desire". Venereal desire is often described as the sexual appetite, and can be understood as a desire for sexual stimulation. However, the increase of the appetite is not essentially associated with an equivalent increase in the capacity to satisfy that sexual desire. Aphrodisiacs can be classified according to their mode of action into two categories. The first type of aphrodisiac acts specifically to increase the libido (*i.e.*, sexual desire). And the second category of aphrodisiac takes action by increasing the ability to indulge in sexual activity (*i.e.*, increasing potency). In theory, an aphrodisiac is strictly an agent which arouses or increases sexual desire, but in practice anything which increases the capacity for sexual enjoyment will tend to increase the appetite and can be considered as an aphrodisiac. There seems to be no limit to the credulity of man in his pursuit of aphrodisiacs, and it has been possible to compile a catalogue of over 500 animal, vegetable, and mineral substances which have, at some time, been evocated for their aphrodisiac properties (Taberner, 1985b). References to such substances have crept into holy texts from the *Kāma Sūtra* and the Bible to the Koran and the literature from Shakespeare and Ovid to Gilbert and Sullivan plays in the twentieth century (Wedeck, 1963).

3. Aphrodisiacs in history: the ancient traditions

The first report discussing sexual pathological disorders dates back 3,000 to 4,000 years, when medical information was passed from one generation to the next through oral poems among the Hindu population (Herman, 1969). These poems were finally inscribed around 2,000 to 1,000 B.C. and one of them (*Samhita of Sushruta*) already reported the pursuit for substances to enhance sexual experience (Sandroni, 2001). The written traditions, preserved in particular cultures and occasionally passed on to other cultures through commerce and other ways, are the source of our knowledge about aphrodisiacs. The diffusion of this knowledge from Egyptians to the Arabs and Greek and so to the Romans was partially interrupted during the Middle Ages because it was considered heretical. Throughout the Renaissance these persecutions came to an end and the intensification of travel and exploration introduced new knowledge to Europe, notably from China, India and the New World. Common aspects have often emerged among different cultures and ethnical groups in the use of plants as aphrodisiacs, even though the application of this knowledge has always had a large magical component (Taberner, 1985a).

The Hindu tradition. Among the numerous classical works in Sanskrit literature on the subject of love, the most famous remain the *Anunga Runga*, written by the poet Kullianmull in the fifteenth century, and the *Kāma Sūtra*, composed by Vātsyāyana some time between the first and fourth centuries A.D. This latter is still celebrated and translations of the book are read

widely all over the world. There is a short chapter in the *Kāma Sūtra* which explains the methods for "attracting others to oneself", including magic rituals, natural remedies of different potencies and general advise (Vâtsyâyana *et al.*, 2000). Some local plants and animals used as ingredients for the recipes have not been identified yet due to their indecipherable Sanskrit names, increasing the mystery concerning the Hindu aphrodisiacs.

Most of the methods reported in the *Kāma Sūtra* to increase the sexual vigor of a man include milk and honey. These have always been recognized as food able to produce endurance and energy, as already described by Greek and Roman writers. These two main ingredients, the immediate source of energy for employing in physical activity, are often combined with generative organs of animals, considered in organotherapy as the basis of sexual potency. Many other recipes are described in the *Kāma Sūtra* but the ingredients are not likely to have anything more than a physiological effect upon sexual desire. The use of highly nutritious food substances (ghee, milk, eggs and honey) in these recipes could have an explanation. In a society where the normal diet tends to be deficient in proteins and essential vitamins, the addition of extremely nutritious supplements would increase the physical vigor and treat a loss of sexual appetite due to a deficient diet. These pseudo-aphrodisiacs bring no benefits to individuals on equilibrated diets and sometimes their over-use can even be damaging.

More directly acting sexual stimulants are described in the seventh part of the *Kāma Sūtra* which is dedicated to the occult practices (Aupanishadika). The components of these stimulant mixtures involve principles used as aphrodisiacs by other cultures. A mixture of powdered thorn-apple seeds, black pepper (*Piper nigrum* L., Piperaceae), long pepper (*Piper longum* L., Piperaceae) and honey, applied on the penis before coitus, is reputed to make a woman subject to the man's will. The four principles have a specific function in the aphrodisiac mixture which has a completely rational basis for its action. The thorn-apple seeds (*Datura stramonium* L., Solanaceae) contain atropine and scopolamine, which are potent tropane alkaloids inducing an initial excitement followed by sedation and hallucinations. These two constituents are adsorbed through the mucous membranes of both the penis and the vagina, and have a central nervous system action producing behavioral effects in both partners (Hostettmann, 2000). The peppers have a counter-irritant or rubefacient action, increasing the blood flow around the area of application. In the man, this local inflammation helps the development and maintenance of an erection, while, in the woman, the irritation of the clitoris increase the sexual desire. Another rubefacient plant, ginger (*Zingiber officinale* Rosc., Zingiberaceae), is described in the *Kāma Sūtra* and employed by man to increase potency. The honey is used for an easier application of the mixture and as a useful lubricant.

During the Middle Ages, other plants of the Solanaceae family such as *Atropa belladonna* L., *Hyoscyamus niger* L. and *Mandragora officinarum* L., were used in Europe for the same hallucinogenic effects as thorn-apple. The mandrake was considered a potent aphrodisiac and a

magical plant at the same time. Its active part was the root, which could resemble the figure of a man (the closer this resemblance, the more powerful the root). The root was an expensive ingredient in magic potions since its collection required a dangerous procedure. On pulling the plant from the ground it was supposed to give vent to a shriek that was instantaneously fatal to anyone within earshot. To overcome this danger, the collectors of mandrake tied a dog to the plant and, after retiring to a safe distance, would call the animal. Medieval witches used mandrake, belladonna and henbane for their pleasure. They applied an ointment of these plants to the moist membranes of the vagina using broomsticks as applicators. This ointment is what allowed witches to fly on their broomsticks to their Sabbaths (Hostettmann, 2000).

The China and Far-east tradition. The Chinese civilization is one of the oldest in the world and its herbal tradition is well-established as confirmed by the pharmacopoeia of Emperor Shen Nung written about 3,000 B.C. This pharmacopoeia already described the virtues of potent preparations, including Indian hemp, the opium poppy and aconite, and is the basis of modern Chinese herbal medicine. In China, this type of health care system is much more developed than Western medicines for social and economic reasons. The Far-east tradition devotes a particular attention to substances which generally promote longevity and particularly restore sexual vigor. One of the most celebrated drugs is the ginseng root (*Panax ginseng* C.A. Mey., Araliaceae) which has been used for centuries in China, Tibet, Korea, Thailand, Cambodia, Laos, Myanmar, Vietnam, and India, where it is reputed to possess the same properties as the mandrake root (*Mandragora officinarum* L., Solanaceae). As for the mystical mandrake, the shape of the ginseng root is similar to the human form and consequently it should be universally beneficial to the body. In fact, the etymology of the name of the plant comes from the Greek language “cure-all” (*panax*: *pana* = all; *axos* = cure), and from the Mandarin language “man-like” (*ginseng*: Anglicization of the Chinese *jin-chen*). In Asia ginseng can be bought in different forms (powder, pills, ointments, tinctures and teas) and is recommended among many other things as an aphrodisiac with the power of rejuvenation. Only recently has ginseng use penetrated to the West. It has turned into a famous preparation for its adaptogen properties and is subject to extensive scientific and medical investigation. The aptitude of ginseng to increase sexual desire or potency has not been demonstrated yet, but it is considered as an aphrodisiac as well because of its non-specific “tonic” effect on the body (Gillis, 1997).

In addition to the humanoid roots, Chinese herbal medicine frequently uses deer antlers and other parts of animals as aphrodisiacs. Organotherapy is widespread in Far-east tradition and the price of many of these items can be very high. The ancient Chinese used animal genitals as a method to increase potency. Tiger and deer penises are still considered a delicacy today. Even the nomadic tribes like the Tartars believed firmly in the effectiveness of animal genitalia for

making aphrodisiacs. They ate the penis of a stallion rather than its testicles because they believe in the efficacy of natural products which merely resemble the penis.

The Near-east tradition. The translations of the early wedge-shaped tablets found by archeologists in Arabian regions reveals that the Babylonians and Assyrians practiced magic rituals to protect themselves against malign spirits and to increase fertility in woman and potency in man. The later Arabic tradition in aphrodisiacs is partially influenced by this earlier knowledge. The Egyptians also practiced magic and the herbalists served also as priests. Many plants used by these healers had beneficial properties recognizable even today, but these properties were understood as the presence of spirits in the plant rather than being due to some active substances. The Egyptian Papyrus Ebers, a medical Egyptian document dated 1,600 B.C., lists prescriptions for various ailments, including impotence. In the latter, baby crocodiles' hearts were mixed with wood oil to the appropriate consistency, and this then smeared onto the man's penis to restore potency (Shah, 2002).

The Greek tradition. Akin to the Arab tradition, the Greek knowledge in aphrodisiacs also derived from the writings of the earlier Mediterranean civilizations. As a result, the magic and supernatural had a great importance in curative practices. The magic love charms of the sorceresses Medea and Circe are just an example of the significance of these practices in Greek mythology. Bizarre ceremonies, associating complex mixtures and the solicitation of gods, were served by priests to cure sexual dysfunctions. These rituals illustrate that irrational human activity can be found throughout the world among tribes and cultures at all level of development, and even contemporary educated man is not immune to these kinds of activities.

The Roman tradition. Even though the Romans produced a developed civilization based on complex laws, the pre-Christian Empire has never been regarded as an exactly ideal example of a society with high moral standards. We contemplate this period as an epoch of violence and sexual excess, and the orgy is still considered as a particularly Roman event. This moral laxity provided the widespread use of a multitude of recipes for love potions, philters and aphrodisiacs of all type. Instructions for the preparation of aphrodisiacs were reported in *Natural History* by Pliny the Elder (23-79 A.D.) (Plinius Secundus, 1991). He collected the aphrodisiac recipes from earlier Roman and Greek traditions and described them in his work even if some were dubious. A strong element of magic pervades many of these recipes, whereas others are simply herbal mixtures. Pliny the Elder wrote of rocket (*Brassica eruca* L., Cruciferae) and nettle (*Urtica dioica* L., Urticaceae) which, among other things, were believed to have aphrodisiac properties. The nettle was used externally for its potent rubefacient action and could serve a useful purpose in sexual stimulation. In fact, the fine hairs on the nettle leaves contain a high concentration of acetylcholine and histamine which produce an inflammatory process. Another

counter-irritant aphrodisiac, but far more dangerous, described by Pliny was the cantharides or Spanish fly (*Lytta vesicatoria* L., Meloidae). Aristotle (384-322 B.C.) was the first person to describe blister beetles as an aphrodisiac. Livia, the Emperor Tiberius's mother, purportedly slipped powdered cantharides into the food of other members of the imperial family to stimulate them into committing sexual indiscretions that could later be used against them.

Xiphion spp. (Iridaceae) and *Orchis* spp. (Orchidaceae) were also recommended herbs. They were believed to have aphrodisiac properties because their root tubers resembled a pair of testicles. In fact, the word "orchid" is derived from the Greek language for testicle.

The Roman tradition included also several plants used to decrease the sexual desire, the anaphrodisiacs. Among other plants, the one most frequently employed for this purpose was the Caecilian lettuce (*Lactuca* sp., Asteraceae) which was considered to have the power of quenching amorous propensities.

The Arab tradition. The Arab civilization, which reached its peak around Baghdad in the thirteenth century, was extremely developed in medicine, chemistry, astronomy and philosophy. Its knowledge was slowly bought back to West Europe by crusaders returning from the Holy Wars. Among the different medical books returned, specific texts containing advice in sexual matters, prescription for sexual disorders, and lists of aphrodisiac drugs and recipes, have miraculously survived the religious intolerance of the Dark Ages. The best-known book on the subject is certainly *The Perfumed Garden*, written in the sixteenth century by Shaykh Umar ibn Muhammed al-Nefzawi and translated by Sir Richard Burton. This book contains contemporary and much earlier references to prescriptions for sexual disorders, and it is probably the most widely known book of sex instruction. Some of its recipes can be even traced to the original Galenic writings ("On the Secrets of Women" and "On the Secrets of Men"). The aphrodisiac mixtures included different drugs, depending on the purpose or the sex of the consumer. For example, drugs that excited the sexual desire of women were: aged olive oil, orchids, garden carrot seed, turnip seed, ash of the leaf of the oleander, dry alum, magpie excrement, powdered willow leaves, and piths of fine dates.

In Arab tradition, the use of hot spices applied to the sexual organs as rubefacients was frequent. Macerated cubeb-pepper or cardamom grains or, alternatively, a mixture of pyrether, ginger and ointment of lilac were recommended by the author of *The Perfumed Garden*. In another chapter, rubefacients were also recommended for increasing the dimensions of small members. The immoderate use of all the known aphrodisiacs and of the strongest hot spices by different Arab populations was presumably due to their apprehension for a premature impotence as a result of the excesses to which polygamy led.

The Arabs were also talented chemists; they were the first to use distillation techniques and were able to use alcohol to dissolve substances insoluble in water. This knowledge helped them

in the preparation of perfumes and essences from plants which had an important function in increasing the pleasure obtainable through sex. The title of *The Perfumed Garden* was probably attributed to the importance of this aspect of sensual pleasure.

4. Assessing the effects of potential aphrodisiacs

Behind the vast mythological component of aphrodisiacs, there also is an honest biological basis for human sexual arousal and a scientific explanation for why many of these compounds give people an effect. Despite the massive popularity of aphrodisiacs throughout history, the medical and scientific community has always been skeptical about them and has tended to conclude that no such substances, in the classical sense, exist. The puritanical values of western culture have turned a blind eye and have only recently begun to explore even medically diagnosed sexual dysfunctions. Consequently, investigations into both sexual stimulation and the effects of specific substances on sexual arousal are limited. Most data collected on the activities of drugs and food has arisen from information published about side effects of drugs designed for other purposes or in the specific area of male erectile dysfunction.

Several conceptual and methodological difficulties should be noted in these types of medical investigation. One of the main problems in proving the existence of substances which can increase sexual desire is the inability of accurately measuring the level of desire. Physiologists have developed methods to measure objectively the level of sexual arousal, but sexual desire can really only be assessed subjectively by the individual, and factors such as conditioning can become vital (Taberner, 1985a). Measurement approaches can usually vary from one study to another, as certain investigators try to assess drug effects on subjective measures of sexual interest or desire, while others emphasize changes in physiological or hormonal indices of sexual response (Rosen and Ashton, 1993).

Supplementary problems involve the potentially confounding results of drug dosage levels and duration of use, characteristics of the patient, and the social and medical context in which a specific drug is taken (Crowe and George, 1989). The conclusions of these investigations are often based upon anecdotal reports or case studies, although the obvious role of reporting biases on the part of patients and physicians (Buffum, 1982). Nonspecific effects of curing with some therapeutic drugs have not been adequately studied even if such remedies are known to enhance sexual performance as a result of improved health or psychological well-being of the individual. Furthermore, the use of double blind controls cannot be overemphasized, because of the significant placebo effect or positive expectations effect of studies in this area (Sonda *et al.*, 1990; Susset *et al.*, 1989). Finally, the sexist bias in this research is evident, as pharmacological effects on female sexuality have been almost completely neglected by most investigators.

5. Aphrodisiacs that increase potency (allow or sustain erection)

An increase in sexual desire caused by aphrodisiacs is not necessarily associated with a corresponding increase in the ability to satisfy this appetite. Drugs that increase the capacity in sexual activity often have a use in clinical medicine to restore the capability of a patient who has suffered from some pathological disorder which has resulted in organic impotence.

5.1. Erectile dysfunction

Many people believe that impotence is a disorder associated with modern civilization. However, preoccupation with potency has been present through the ages (Shah, 2002).

The term "impotence" is derived from the Latin word *impotentia*, which literally translated means lack of power. It has traditionally been used to signify the inability of the male to attain and maintain erection of the penis sufficiently long to permit satisfactory sexual intercourse. This term, together with its pejorative implications, has often generated confusion in both clinical and basic science investigations, so it was recently replaced by the more precise term "erectile dysfunction" (NIH, 1993).

For years, psychological factors were implicated as the main cause of impotence, but during the last decade there has been an important change in the management of sexual dysfunction. This revolution was essentially due to the improved understanding of erectile physiology and to the development of new and effective medical therapies. The number of men affected by erectile dysfunction has reached approximately 10 to 20 million in the United States (NIH, 1993). Even though the loss of erectile function is not an inevitable consequence of normal aging, it becomes more frequent with age. Recent investigations reveal that up to 70% of 70 years old males have some dysfunction and 50% have moderate or severe problems (Colpo, 1998).

In most men, erectile dysfunction is thought to be the consequence of organic deterioration, especially circulatory insufficiency (Krane *et al.*, 1989). The usual origin is an organic factor or disease, such as hypertension, hypercholesteremia, vascular and heart diseases, diabetes mellitus, surgery and trauma, side effects of medications or neurodegenerative disorders, and the use of tobacco. Psychological problems also give an important contribution to the weakening of sexual performance, diminishing self-esteem, and disrupting personal relationships (Feldman *et al.*, 1994).

An erection is a hemodynamic balance between inflow and outflow of blood within two chambers, called the corpora cavernosa (Figure 0-4). These cavities, which run the length of the organ, are filled with spongy tissue, and surrounded by a membrane, called the tunica albuginea.

The spongy tissue contains smooth muscles, fibrous tissues, spaces, veins, and arteries. Erection begins with sensory and mental stimulation. Impulses from the brain and local nerves cause the muscles of the corpora cavernosa to relax, allowing blood to flow in and fill the open spaces. The blood creates pressure in the corpora cavernosa, making the penis expand. The tunica albuginea helps to trap the blood in the corpora cavernosa, thereby sustaining erection. Erection is reversed when muscles in the penis contract, stopping the inflow of blood and opening outflow channels. The relaxing of the muscles of the corpora and penile arterial vessels is mediated by nitric oxide (NO), which is synthesized in the nerve terminals innervating these muscles. Any problem in blood inflow and outflow can be the origin of erectile dysfunction.

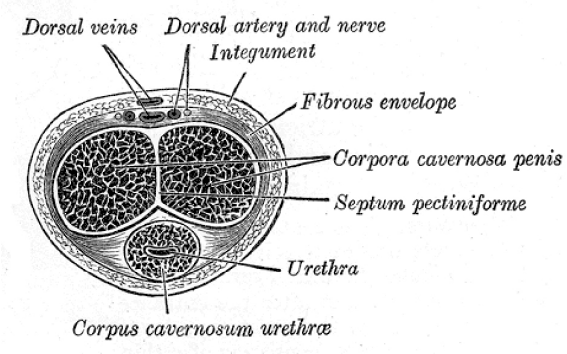


Figure 0-4

Transverse section of the penis.

Normal neurologic innervations of an intact vascular tree and normal sexual desire are essential factors for a man to achieve an erection. When one or more of these factors fails or is deteriorated, an erectile dysfunction can be developed by the individual. Since 90% of all patients with impotence have a physiologic problem, that is where most of the treatment options are focused (Colpo, 1998). Current treatments of erectile dysfunction include oral medication, intracorporal injections, intraurethral applications, vacuum pumps, and penile prostheses.

5.2. Oral medication

Three oral medications are well-known for their ability to cause and sustain an erection: Spanish fly, yohimbine and the phosphodiesterase inhibitors. Spanish fly is an ancient natural aphrodisiac which has fallen into disuse for the treatment of erectile dysfunction because of its numerous toxic effects. Yohimbine is an indole alkaloid extracted mainly from an African plant (Pausinystalia yohimbe PIERRE ex BEILLE, Rubiaceae) which has recently been the focus of new public interest. As more and more information is gained on the mechanism underlying penile erection, more drugs are being developed to treat erectile dysfunction. The most promising of these synthetic medications are the phosphodiesterase inhibitors which are effective in treating both organic and psychological impotence.

5.2.1. Yohimbine

Yohimbine is an indole alkaloid (Figure 0-5) with a competitive antagonist activity selective for the α_2-adrenergic receptor (Murburg *et al.*, 1991).

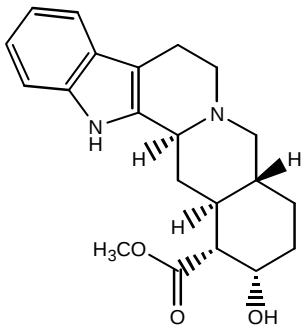


Figure 0-5
Structure of yohimbine.

It comes from the bark of the West African tree *Pausinystalia yohimbe* PIERRE ex BEILLE (Rubiaceae) (Synonym: *Corynanthe yohimbe*) and it was first isolated in 1896. The bark of the tree is traditionally used in African and West Indian medicine as an aphrodisiac with the ability to increase sexual potency and virility. Afrodex®, a drug containing yohimbine hydrochloride, methyl testosterone and *Strychnos nux-vomica* L. (Loganiaceae), was claimed to have aphrodisiac powers and remained on the African market until 1973 (Sonda *et al.*, 1990). Renewed interest in yohimbine for erectile dysfunction has prompted several new investigative trials, including chemical ones. Some indications are that in certain cases yohimbine can improve sexual performance (Ernst and Pittler, 1998), but generally it has limited efficacy. The American Urological Association in its clinical guidelines recently states that "… yohimbine

does not indicate a significant role in the treatment of organic erectile dysfunction… reported benefits have been modest and there is a pronounced placebo effect" (Montague *et al.*, 1996). Despite studies which show that yohimbine is generally well tolerated with few and benign side effects, other reports illustrate its potential risks on certain individuals. Common adverse effects of yohimbine include increased blood pressure, tachycardia, anxiety, and manic reactions (Grossman *et al.*, 1993; Gurguis and Uhde, 1990; Holmberg and Gershon, 1961; Lacomblez *et al.*, 1989; Onrot *et al.*, 1987; Price *et al.*, 1984; Teloken *et al.*, 1998). This drug is still used for men with a psychological component to their erectile dysfunction, or for those who absolutely refuse any other type of therapy. Most men receive prescriptions for this medication, but it is available over the counter at health food stores or it is sold on the Internet. For more chemical and pharmacological details on yohimbine, see chapters **III.1.2** and **III.1.3**.

5.2.2. Spanish fly

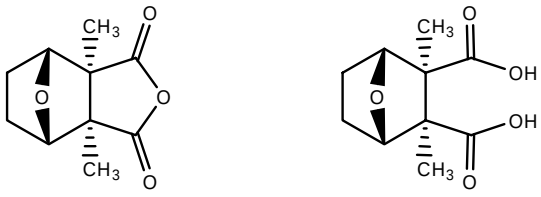
Spanish flies or cantharides are the common names for a variety of blister beetles, usually black or bronze-green, mostly elongate and cylindrical (18 to 25 mm), belonging to the family Meloidae (order Coleoptera). Cantharides is generally specified as the Eurasian *Lytta vesicatoria* L. (syn. *Cantharis vesicatoria*) (Figure 0-6), or as other genera, particularly *Mylabris* and *Epicauta*. The beetles are most commonly found in southern Europe, Africa, and Asia. Adult beetles are phytophagous, feeding especially on plants of the families Amaranthaceae, Asteraceae, Leguminosae, and Solanaceae. Some American species are pests, devastating potato, tomato, beet and clover crops. The larvae, on the other hand, are considered beneficial because they feed on grasshopper eggs. When disturbed, the beetles release an irritating substance called cantharidin (3α,7α-dimethylhexahydro-4,7-epoxyisobenzofuran-1,3-dione) from the joints between their leg segments.

Figure 0-6

Lytta vesicatoria L. (syn. *Cantharis vesicatoria*), Meloidae.

Preparations containing the dried beetles have been official in most European and American Pharmacopoeias, and still linger on in veterinary medicine as blistering and wart-removing agents. The beetles for these preparations are collected before sunrise, while unable to use their wings, by shaking them from the trees on to cloth spread underneath.

The main active principle of cantharides is cantharidin (Figure 0-7), a crystalline lactone, of which good specimens of the beetle yield from 0.4 to 1.1%. It exists in the beetles mainly in the free state, but a varying proportion is combined in the form of the potassium salt of cantharidinic acid. Cantharidin is a vesicant and a mucosal irritant causing intraepidermal blisters. It was formerly topically used as counter-irritant and internally as diuretic and for impotence. Due to its numerous toxic effects, cantharidin has fallen into disuse for most legitimate medical purposes. The toxic dose in humans can be as little as 3 mg and the fatal dose is only 32 mg (Taberner, 1985b). Cantharides also contain 10 to 15% of fat.



cantharidin cantharidinic acid

Figure 0-7

Structures of cantharidin and cantharidinic acid.

Humans were quick to attribute aphrodisiac properties to cantharidin. As far back as the third century B.C., Aristotle had already described the properties of blister beetles, and Pliny the Elder (23-79 A.D.) reported the aphrodisiac virtues of these insects in his work *Natural History*. Tacitus, in his *Annals of Imperial Rome*, mentioned that cantharides were available and used by the Emperor Tiberius's mother, Livia, to stimulate the sexual appetite of other members of the imperial family. During the kingdom of Louis XIII (1610-1643), a preparation containing cantharides was commercialized by the minister Armand Jean du Plessis (Cardinal de Richelieu) under the famous name of "bombons à la cantharide du Cardinal" (Hostettmann, 2000). Madame de Pompadour apparently used tincture of cantharides to regain and maintain the sexual attentions of King Louis XV. In 1772, the Marquis de Sade, the infamous French writer of novels and short stories characterized by a preoccupation with sexual violence, was

tried in court for poisoning several prostitutes. He had, in secret, given them cantharides in the hope that it would arouse them sexually.

The aphrodisiac reputation of cantharides probably rests upon the ability to cause irritation of the urethra with resultant vascular congestion and inflammation of the erectile tissue in male or female genitalia, a sensation that may be interpreted as enhanced sexuality by some (Till and Majmudar, 1981). Besides its long and colorful history, perpetuated by a reputation as an aphrodisiac, cantharides and their active principle cantharidin are famous for their toxicity as punctuated by numerous reports of poisoning (Fisch *et al.*, 1978; Harrisberg *et al.*, 1984; Karras *et al.*, 1996; Marcovigi *et al.*, 1995; Polettini *et al.*, 1992; Tagwireyi *et al.*, 2000; Till and Majmudar, 1981). Many of the toxic effects of cantharides are directly related to the vesicant properties of cantharidin. It causes blistering of mucus membranes upon contact: hemorrhagic mucositis of the mouth, esophagus, and stomach is a frequent cause of death from the drug. Gross genitor-urinary hemorrhage is another common occurrence and results from tubular necrosis and glomerular damage as well as from blister formation in the lower urinary tract. Some references mention cases of persistent erection or priapism, which may be extremely painful, particularly if urination becomes impossible (Karras *et al.*, 1996).

Unfortunately, the myth of Spanish fly as an aphrodisiac has outlived its medical utility. It is debatable as to whether or not commercial preparations of Spanish Fly sold in western sex-shops and through the mail actually contain cantharidin. The high toxicity may well prevent many producers from really adding cantharidin to “love potions”. Such products are usually labeled “placebo” and any cantharidin that is present is contained in homeopathic dilutions. However, other preparations containing cantharidin in concentrations capable of causing severe toxicity are available illicitly on the black market or from countries where the controls are less stringent.

There is another potential risk of cantharidin poisoning in African countries where beetles are considered delicacies and where ingestions of frogs or birds which have eaten the beetles may also cause toxicity (Eisner *et al.*, 1990).

5.2.3. Phosphodiesterase inhibitors

As information is progressively gained on how the relaxation process takes place in the penis, more drugs are being developed to treat erectile dysfunction. The phosphodiesterase type 5 (PDE5) inhibitors are new drugs which affect local regulation of erectile function by potentiating the effects of nitric oxide (NO).

The first developed and consequently the most famous is sildenafil (Figure 0-8), the active principle of the oral pill Viagra®, produced by the Pfizer Corporation, and approved by the FDA in early April 1998. This active compound is effective in treating both organic and psychological impotence (Boolell *et al.*, 1996).

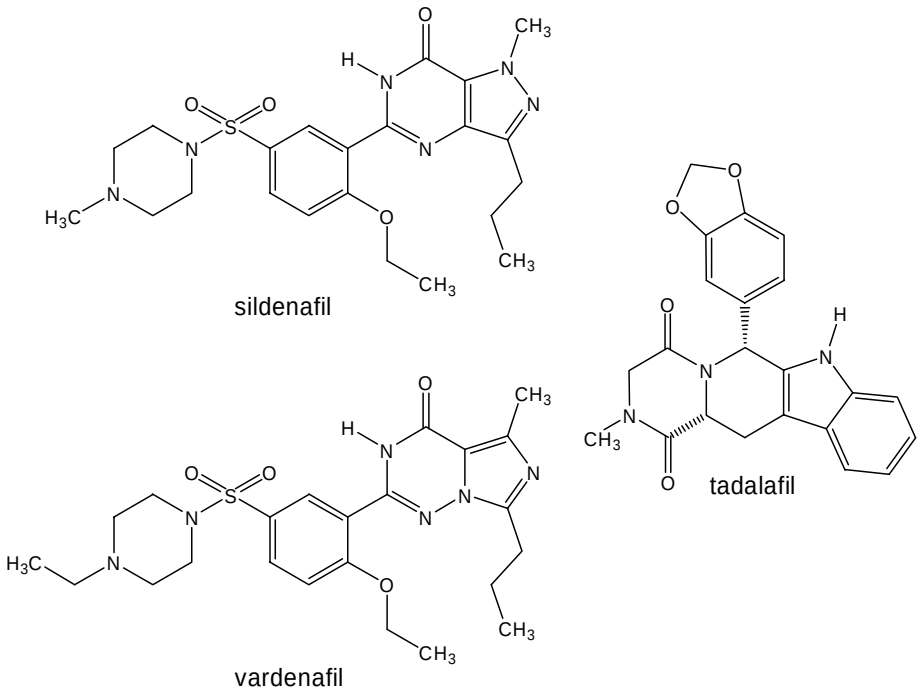


Figure 0-8

Structures of three phosphodiesterase type 5 inhibitors.

As already explained in previous chapters, penile erection is a hemodynamic process involving relaxation of smooth muscle of the corpus cavernosum and its associated arterioles. This relaxation process results in an increased flow of blood into the trabecular spaces of the corpora cavernosa (Andersson and Wagner, 1995). Smooth muscle relaxation is mediated by NO which, on sexual stimulation (visual or otherwise), is released in the nerve terminals of parasympathetic non-adrenergic, non-cholinergic neurons in the penis and also by the endothelial cells of blood vessels and corpora cavernosa (Burnett *et al.*, 1992). NO activates guanylate cyclase which increases the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). This latter provides the signal which leads to relaxation of smooth muscle of the corpus cavernosum and the penile arterioles. The erection caused by this relaxation ceases after a while because cGMP is hydrolyzed to guanosine 5'-monophosphate (GMP) by cyclic nucleotide phosphodiesterase (PDE) isozymes (Beavo, 1995). Of the PDE isozyme families known, only PDE5 and PDE6 are specific for cGMP as a substrate. PDE3 and PDE4 are specific for cyclic adenosine monophosphate (cAMP), and PDE1 and PDE2 hydrolyze both cGMP and cAMP. Therefore, substances that inhibit cGMP hydrolysis increase the cGMP signal, enhancing the relaxation of smooth muscles in the corpus cavernosum and

facilitating penile erection. Sildenafil is a potent inhibitor of the cGMP-specific PDE5 found in corpus cavernosum (Figure 0-9).

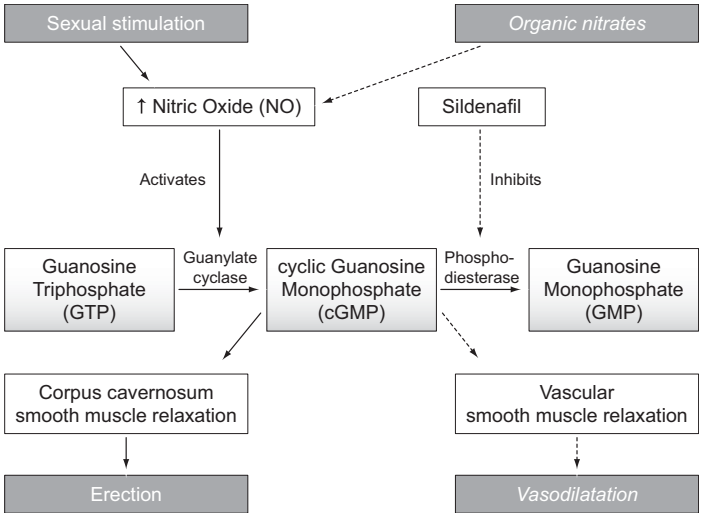


Figure 0-9

Action of sildenafil (Viagra®) in erectile dysfunction (Drewes *et al.*, 2003).

Sildenafil has to be taken 1 hour before desired intercourse and then, ½ to 1 hour later, the sexual stimulation must be performed to bring on the erection. If no stimulation occurs, nothing will happen. Side effects can include facial flushing, mild headaches, diarrhea and mild disturbance of the vision (blue haze). Sildenafil has not to be administrated to patients with heart problems taking nitrate medications because of the severe potentiation of vasodilatory effects.

Two other potent, selective, reversible PDE5 inhibitors, tadalafil (Cialis®) and vardenafil (Levitra®) (Figure 0-8), respectively produced by Lilly-ICOS and Bayer Pharmaceutical Company, are under regulatory review in Europe, the United States and other countries. In both chemical and PDE subtype selectivity profiles, tadalafil differs markedly from sildenafil and vardenafil. Tadalafil is approximately 780 times more selective for PDE5 than PDE6 while vardenafil and sildenafil are, respectively, only 2.9 and 6.8 times more selective (Brock, 2002). Although these *in vitro* selectivity ratios cannot be extrapolated to clinical findings with each PDE5 inhibitor, color visual disturbances occur in up to 11% of sildenafil patients, in a small minority of vardenafil patients and in only 0.1% tadalafil patients. Compared with sildenafil and vardenafil, tadalafil also exhibits a prolonged plasma residence ($t_{½}$ 17.5 hrs) and prolonged therapeutic response.

5.2.4. Alternative herbal remedies

The commercialization of the synthetic "love drug" Viagra® has not only captivated the public imagination, but has led to the reconsideration of classical natural aphrodisiacs for their use to combat impotence, and to the search for a new natural product which can truly compete with Viagra®. Since the reassessment of "old" natural products such as yohimbine and cantharidin has not given new significant results, investigators have directed their researches at aphrodisiacs used in various traditional medicines. A lot of work has been done to trace their history, to examine herbs as folklore traditional healing, and to summarize the latest scientific research on their many benefits and the ways potential hazards, if any, can be avoided (Adimoelja, 2000).

Vuka-vuka. With the explosion of web-based sales, it is now possible to buy herbal remedies from around the world, including *vuka-vuka*, the "African natural Viagra®". This traditional preparation originates from Zimbabwe and has become famous worldwide after a programme of the American CNN in 1998. In Zimbabwe, *vuka-vuka* is a popular drug used by a high proportion of men. In Ndebele, one of the languages of Zimbabwe, *vuka-vuka* means "wake-up, wake-up". The aphrodisiac preparations are sold at different markets in Harare (Mbara market) and Bulawayo by traditional healers. There are two forms of *vuka-vuka*, one an herbal remedy and the other a concoction containing cantharidin extracted from *Mylabris* spp. This latter has already been the cause of several health problems in Zimbabwe and can be potentially dangerous for tourists attracted by experimenting with new aphrodisiacs. The herbal preparation has different constituents, depending on the traditional healer who formulates it. In general, three drugs are common to all mixtures and have equal amounts: the root bark of *Mondia whiteii* Skeels (Asclepiadaceae), the roots of *Albizzia antunesiana* Harms (Leguminosae), and the stem bark of *Ozoroa insignis* Delile (Anacardiaceae). Three other plants are traditionally used in the formulation of *vuka-vuka*, but they are not always present: the stem or root bark of *Pouzolzia hypoleuca* Wedd. (Urticaceae), the roots of *Elephantorrhiza goetzi* Harms (Leguminosae), and the stem bark of *Cassia singueana* Delile (Leguminosae). The efficacy of these plants in the cure of impotence have not been demonstrated yet.

Tribulus terrestris. *Tribulus terrestris* L. (Zygophyllaceae) is an annual creeping herb growing on roadsides and hills in China, Japan, Korea, the western part of Asia, the southern part of Europe and Africa. It has long been used in the traditional Chinese and Indian systems of medicine for the treatment of various ailments and is popularly claimed to improve sexual function in man. Several researchers have presented evidence that protodioscin, a compound present in the *Tribulus terrestris* L. extract, can improve sexual desire and enhance erection (Adaikan *et al.*, 2000; Adimoelja, 2000). The activity of the plant has been clinically proved

(Miller, 1988). It is considered as a neurosteroid which acts centrally as a γ-aminobutyric acid antagonist to facilitate sexual function. The mechanism of the increase of DHEA after a constant administration of the extract of *Tribulus terrestris* L. still needs some clarification, however some studies provide evidence for the claimed role of this plant as an aphrodisiac in traditional medicine (Adaikan *et al.*, 2000; Adimoelja, 2000; Gauthaman *et al.*, 2002).

Maca. *Lepidium meyenii* Walp. (Brassicaceae), commonly known in South America as *maca*, is a nutritionally valuable Peruvian plant and is traditionally used in the Andean region for its supposed properties to improve energy, fertility in both sexes and male potency (Cicero *et al.*, 2001; Muhammad *et al.*, 2002). *Maca* is a crop which is found only in a very restricted area of central Peru, in the agro-ecological zone above 4000 m where low temperature and strong winds limit other crops. It can be successfully cultivated outside its current natural habitat giving edible and high nutritive (especially proteins and minerals) subterranean parts (hypocotyl). There is no reliable scientific evidence to confirm a positive effect of *maca* on an increase of sexual performance, but recent studies have shown that oral administration of a lipidic extract from *L. meyenii* increases the number of complete introductions and the number of sperm-positive females in normal mice and decreases the erection latency in male rats with erectile dysfunction (Cicero *et al.*, 2001; Zheng *et al.*, 2000). Besides this scientific evidence, further systematic approaches are needed to find which constituent of the plant is responsible for the observed effect on the sexual performance parameters.

Catuama® and *muira-puama*. Catuama® is a Brazilian herbal medicine recommended for certain disorders, including physical and mental fatigue, neuromuscular asthenia and general weakness (Antunes *et al.*, 2001). Catuama® consists of a mixture of crude hydroalcoholic extracts of four medical plants: *Paullinia cupana* H.B. et K. (Sapindaceae; guaraná), *Trichilia catigua* A. Juss. (Meliaceae; catuaba), *Zingiber officinale* Rosc. (Zingiberaceae; ginger), and *Ptychopetalum olacoides* Benth. (Olacaceae; muira-puama).The individual components have also been used, separately, for centuries, to cure different complications. However, more recently, it is the combination of the four plants that has been promoted as an aphrodisiac. Latest investigations have shown that Catuama® relaxes various arterial vessels by a mechanism involving the release of nitric oxide (NO) (Calixto and Cabrini, 1997). This relaxing activity was attributed to active principles present mainly in *P. cupana*, *T. catigua* and *Z. officinale*. Since relaxation of corpus cavernosum is a key step in penile erection, the effects of Catuama® and the extracts of single plants were examined on isolated rabbit corpus cavernosum preparations using a bioassay cascade (Antunes *et al.*, 2001). In these experiments, Catuama® and each of its plant constituents showed dose-dependent relaxation of the rabbit isolated corpus cavernosum. *T. catigua* extract caused a prolonged and sustained relaxation, while the other

extracts produced short-lived relaxations. Of the four extracts assayed, *P. cupana* was the most effective in causing relaxation of smooth muscle.

The wood and bark of *Ptychopetalum olacoides* Benth. and *Ptychopetalum uncinatum* Anselmino (Olacaceae) make up the herbal remedy known in Brazil as *muira-puama*. These plants have been traditionally used for different purposes, including the treatment of erectile dysfunction (Drewes *et al.*, 2003). Even though more investigations are needed to prove the efficacy of *muira-puama*, a human trial carried out by a French group showed the herbal remedy to be effective in improving libido and treating sexual impotency (Waynberg, 1990).

It has to be noted that the vernacular name of *T. catigua*, *catuaba*, is the name given to a popular herbal medicine from Brazil. Plants of different genera have been referred to as *catuaba*, and these have been attributed with aphrodisiac and tonic properties (see chapter IV.1.1).

5.3. Intracorporal injections and intraurethral applications

In the early 1980s, treatment of erectile dysfunction took a whole new turn with the discovery of vasoactive drugs which injected into the penis could produce a rigid erection. The most common agents used today include papaverine, prostaglandin, and phentolamine, either alone or in combinations with each other (Figure 0-10). In general, 75% of men with erectile dysfunctions have a good response to injection therapy. However, patients must be carefully instructed in the method of penile injections in order to avoid complications.

Papaverine. Papaverine is a potent direct smooth muscle relaxant isolated from the white poppy, *Papaver somniferum* L. (Papaveraceae). This plant yields opium, the dried latex of the immature poppy capsule, a mixture of twenty-six alkaloids, including morphine, codeine, laudanine, thebaine, and papaverine. It is the earliest known painkiller and archeological evidence suggests that opium poppies were grown as a medical and psychoactive drug during the Neolithic and Bronze Ages.

Virag was the first to discover that an injection of papaverine in the corpus cavernosum produced erections by causing arterial vasodilatation and smooth muscle relaxation (Virag, 1982). Since that time, other substances have been identified. The most dangerous complication that can occur with papaverine injections is priapism (Lomas and Jarow, 1992). This is a full, firm erection that lasts more than 4 hours, becoming quite painful and if not detumesced can cause permanent damage. Other complications can include scarring, bleeding, and bruising at the injection site, pain, and infection (Colpo, 1998). Scarring may occur as a result of repeated injections in a small area or by the acidity of the injection solutions. Because of the high

incidence of these side effects, the use of papaverine as a single agent has fallen out of favor. It is commonly used in a tri-mix combination, including prostaglandin and phentolamine.

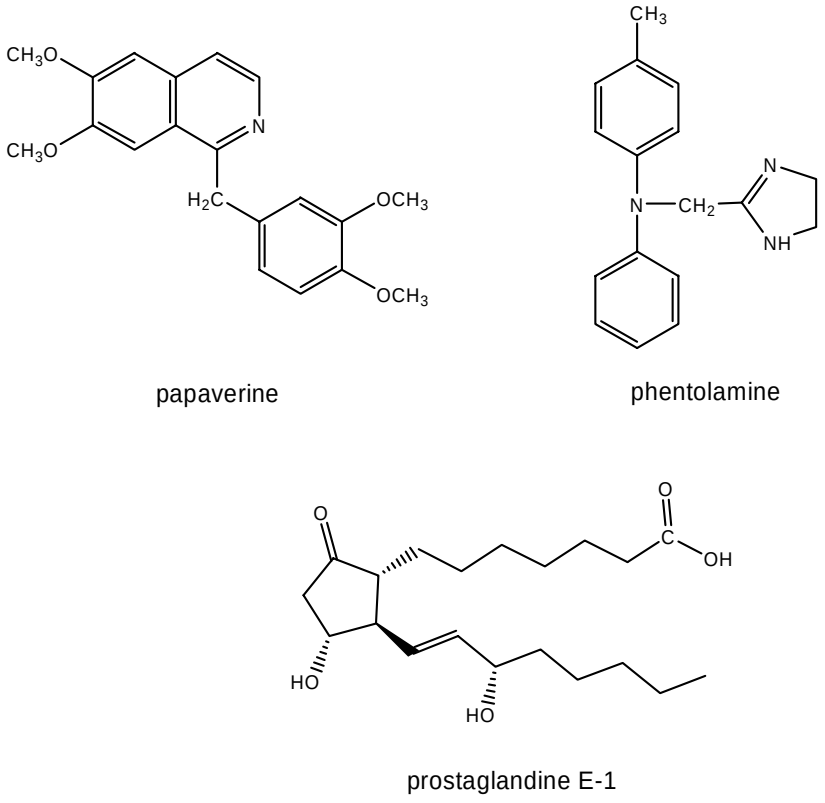


Figure 0-10

Structures of papaverine, phentolamine, and prostaglandin E-1.

Prostaglandin. Prostaglandin (PGE-1) is a naturally occurring agent that induces smooth muscle relaxation. It is released by the prostate during ejaculation and, as an injectable agent, has the advantage of being metabolized quickly within the penis. This decreases the incidence of priapism and scarring. Used as a single agent, PGE-1 has the disadvantage of causing penile pain, which occurs in up to 30% of patients (Schramek *et al.*, 1990).

A related substance of prostaglandin, alprostadil, is used as an intraurethral drug. This drug, in a pellet form, is inserted into the end of the penis through an applicator and absorbed through the urethral wall into the corpora, causing an erection 10 min later. This is an innovative system because it is simple and non-invasive. However, some disadvantages, such as burning of the urethra upon application, pains similar to that of prostaglandin injections, possible light-headedness or syncope after application, erections which are not so rigid and cost of the medication, can discourage patients from using this intreurethral medication (Colpo, 1998).

Phentolamine. Phentolamine in an α-adrenergic antagonist that modulates the sympathetic nervous system. Its employment as a single agent is very erratic. When used in combination with papaverine and PGE-1, phentolamine potentiates the effect of these latter and reduces their incidence of pain (Govier *et al.*, 1993).

6. Aphrodisiacs that increase sexual desire

The aphrodisiacs discussed in this chapter are oral pharmacological agents with potential to directly influence the motivational and/or pleasure components of sexual response. In general, these types of aphrodisiacs act at the level of the central nervous system (CNS) by changing specific neurotransmitters or specific sex hormone concentrations. Some of them can be effective in both sexes, although those acting through an increase in hormone concentration are often sex-specific.

The role of neurotransmitters and hormones has been extensively investigated and there is growing evidence that some of them may have positive effects on sexual performance. Certain neurotransmitters and peptides such as dopamine, oxytocin and adrenocorticotrophic hormone (ACTH), have facilitatory effects on sexual behavior while γ-aminobutyric acid (GABA), endogenous opioids and norepinephrine appear to be inhibitors. The complex role of neurotransmitters in the control of sexual behavior has led researchers to investigate a broad range of receptor agonists (stimulating agents) and antagonists (blocking agents). Some of the traditional aphrodisiacs also work via peripheral and central neurotransmitter action. The alkaloid yohimbine, for example, stimulates the peripheral and central nervous system by acting on α-adrenoceptors (Feuerstein *et al.*, 1985).

Dopamine agonists. The neurotransmitter dopamine also stimulates copulatory behavior. There is considerable evidence for prosexual effects of dopamine agonists in various nonhuman species, but the first indication of these affecting humans came from early studies of dopaminergic drugs in the treatment of Parkinson's disease. In fact, soon after L-dopa became clinically available, a number of clinical reports appeared of hypersexuality in patients receiving drug treatment (Rosen and Ashton, 1993). In 1978, E. Brown and coworkers reported that four out of seven men taking L-dopa had increased sexual interest or activity. Based on intensive neurologic and psychiatric assessments before and after treatment, they concluded that sexual drive changes were unrelated to improved physical or mental health in these patients (Brown *et al.*, 1978).

Clinical studies on L-dopa and other dopaminergic drugs such as apomorphine, nomifensine and bupropion have shown increased sexual desire and arousal in patients with erectile

dysfunction or inhibited sexual desire. Interestingly, clinical work on nomifensine and bupropion, two atypical antidepressant agents, was conducted on both male and female patients and were found to be active in both sexes (Rosen and Ashton, 1993). Even though these studies have reported positive results, the high rate of adverse side effects limits the clinical usefulness of these drugs as well as other dopaminergic agents of this type. Despite this, some entrepreneurial individuals in the US and Europe are making a lot of money out of the supposedly aphrodisiac effects of L-dopa.

α-Adrenergic drugs. The most famous active principle having a prosexual effect and acting as a α-adrenergic antagonist, is certainly yohimbine (see chapter III.1.3). Even though different studies suggest that yohimbine treatment has the capacity to positively affect sexual desire and performance, at least in some subjects other studies report that more than half of all patients have shown little or no benefit from the drug. Based upon investigations of yohimbine's effects on mounting behavior in male rats, Clark and coworkers suggested that α-adrenoceptor stimulation directly increases libido (Clark *et al.*, 1984). Unfortunately, these results failed to be confirmed by another study carried out by Chambers and Phoenix on rhesus monkeys (Chambers and Phoenix, 1989).

Serotonergic drugs. Serotonin is generally viewed as an inhibitory neurotransmitter in the control of sexual drive (Foreman and Wernicke, 1990). However, several serotonergic drugs such as trazodone, clomipramine, and fluoxetine, have been reported to enhance sexual activity. Trazodone, for example, a well known antidepressant, inhibits serotonin re-uptake and several clinical trials have reported increased libido in male and female patients treated with the drug. Changes in sexual desire are apparently unrelated to the antidepressant effects of the drug. Nevertheless, despite potential benefits these drugs have serious side effects (sedation, blurred vision, dry mouth, fatigue, constipation, headaches) and some patients may find prolonged erectile stimulation embarrassing.

7. The commercial accessibility of aphrodisiacs and their dangers

The long pathway from advertising aphrodisiacs as magic-religious charms to witchdoctors, herbalism, pseudo-scientists and Viagra® is a fascinating one. Throughout the ages, quacks have used aphrodisiacs to exploit vulnerable victims, 30% of whom through the power of suggestion have achieved sexual success from potions, powders and sexual pomades (Renshaw, 1978).

All through history, pseudo-healers and charlatans have sought success in marketing what they claim to be sex-potions and they have often succeeded in selling their miracle drugs. Herbal medicine is an ancient and respected science which has led to the discovery of many of the effective drugs used clinically today. Ancient herbal texts have also provided the basis of modern medicine. The genuine herbalists have always had complete faith in the value of their treatment and their objective has been at all times to help their patients. In opposition, the quacks have unashamedly attempted the exploitation of their clients by offering remedies which they know to be ineffective and selling them at inflated prices. Preparations which are claimed to increase sexual desire or virility are clearly going to provide lucrative benefits for these charlatans who trade upon the credulity and ignorance of their clients. Taberner in his eminent book on aphrodisiacs clearly reported this conception about herbalism and quackery:

"There is a grey area between herbalism and quackery into which many of the reputated aphrodisiac preparations fall. The simplest means of differentiating between the two is to consider the intent of the seller as being the important criterion. Once a herbal remedy has been shown conclusively to be a little or no worth, then it becomes quackery to continue to sell it with that knowledge." (Taberner, 1985a).

In the 21st century, obsession with sexual performance is an international phenomenon and many entrepreneurs try to obtain large profits from it as shown by the widespread trade of all sexually related products, including aphrodisiacs. Quackery is still a common practice in our time and charlatans have gained new means of spreading their impostures. While in the past, advertising aphrodisiacs was primarily by word of mouth, today internet advertising, radio airtime, and commercial-packed television broadcasts are potent means for marketing "wonderful" sex-remedies. The power and the high responsibility of these media in the divulgation of dubious health claims and in the commercial promotion of certain drugs, is illustrated by the explosion of the Viagra® phenomenon in 1998. Led by an immense marketing strategy orchestrated by the manufacturing company Pfizer and with the aid of news coverage, Viagra® has become one of the best-selling drugs in modern medical history. The wide media attention for Viagra® has created a new massive public interest in aphrodisiacs and remedies for

erectile dysfunction, especially in the so-called herbal alternative products. Although many of these products sold are labeled as “natural”, this does not ensure the product’s safety or efficacy. Many of the reputed aphrodisiacs described in this work could be safe drugs or dangerous poisons. In fact, there is no real distinction between a drug and a poison; many clinically used drugs can be toxic at doses only slightly above the recommended therapeutic dose. There is also a widespread and unfortunate misconception that a drug of natural origin must intrinsically be good and cannot cause any poisoning, but people often forget that the most deadly venoms are from nature. It is quite likely that many cases of accidental self-poisoning arise from this erroneous idea.

Toxic and lethal cases involving individuals who seek new sexual experiences using chemical and herbal aphrodisiacs are constantly reported throughout the world. Upon exposure, the clinical toxicity may vary from mild to severe and may even be life-threatening.

During February 1993-May 1995, the New York City Poison Control Center was informed about the onset of illness in five previously healthy men after they ingested a substance marketed as a topical aphrodisiac; four of the men died (MMWR, 1995). The deceased died from cardiac dysrhythmia due to inappropriate use of the purported aphrodisiac which was found to contain bufadienolides, naturally occurring cardioactive steroids that have digoxin-like effects. The product marketed as an aphrodisiac was sold under names such as “Stone”, “Love Stone”, “Black Stone”, and “Rock Hard” and was available in grocery stores and smoke shops and from street vendors. In addition to bufadienolides, the active ingredients of these aphrodisiacs, extracted from the Bufo toad (*Bufo marinus* L.) skin and glands, contained bufotenin and its O-methylated derivative, a putative hallucinogen congener of serotonin. The same cardioactive steroids have been found as the active ingredients in *Chan Su*, a Chinese patent medicine used as a topical anesthetic and cardiotonic agent. Cardioactive steroids, including bufadienolides, have a narrow therapeutic index, and unintentional therapeutic intoxication is well documented.

As already reported in the previous chapter, the aphrodisiac “Spanish fly” is another well-documented source of poisoning in different countries (Fisch *et al.*, 1978; Harrisberg *et al.*, 1984; Karras *et al.*, 1996; Marcovigi *et al.*, 1995; Polettini *et al.*, 1992; Tagwireyi *et al.*, 2000; Till and Majmudar, 1981).

Due to increased morbidity and mortality, poisonings associated with the use of herbal remedies have raised universal attention in the last few years (Deng, 2002). But unfortunately, in daily practice, many cases of herbal poisoning were not diagnosed or treated correctly, and therefore, more information about the toxicology of herbal products is urgently needed. Lack of regulation, easy accessibility, contamination, adulteration and toxic potential are some of the dangers deemed to be associated with aphrodisiac poisoning. Of these factors, contamination, adulteration and natural toxic substances are three main risk factors connected with the

aphrodisiac product itself, which may potentially cause poisoning. Herbal aphrodisiacs may be contaminated with pesticides, moulds and/or heavy metals in the botanical product, and by minerals and/or prescription drugs in the final medicines. Herbs may also be adulterated with western medicines, such as steroids, hormones, CNS stimulants, *etc*. Recently, the FDA issued a warning on an all-natural herbal product claiming to increase the sexual experience for both men and women, and found to contain Viagra® (Mitka, 2003). The incriminated product, Vinarol®, was sold over the counter and through the Internet, at a cost that ranged from $6 to $10 per tablet. A spokeswoman of the company which commercialized Vinarol® claimed either employee tampering was responsible, or a shipment of herbs from China was already tainted with sildenafil.

Since herbal poisoning can result from contamination, adulteration or natural toxins, qualitative and quantitative analyses to delineate the incriminating components should be undertaken. Scientific research conducted to investigate the biological activity, safety, and efficacy of aphrodisiacs would be of great benefit to patients and the health community.

III. Qualitative and quantitative determination of yohimbine in authentic yohimbe bark and commercial aphrodisiacs

1. Introduction

1.1. Yohimbe: the revival of an old aphrodisiac

Since its commercialization, Viagra® has focalized huge media attention and created massive public interest in the availability of so-called aphrodisiacs and remedies for erectile dysfunction. This interest has had a knock-on effect and has led to a resurgence of sales in more easily-available herbal supplements‡ (LaFrance *et al.*, 2000), in particular those containing yohimbe (Sunderland *et al.*, 1999). The importance of this phenomenon has been reported, but also extended by the existence of thousands of websites pretending to have discovered miraculous natural mixtures able to increase sexual desire (Figure 0-1).

One of the main recurrent ingredients of these commercialized products is the dried bark of *Pausinystalia yohimbe* PIERRE ex BEILLE (Rubiaceae) (Synonym: *Corynanthe yohimbe*) (Betz *et al.*, 1995), a tree native to the coastal forests of Central Africa and distributed from Southeast Nigeria to the Congolese Mayombe region. *P. yohimbe* is a fast-growing tree but does not reach a great diameter (max. of ca. 50 cm) and has been referred to as "common". This bark has long been considered an aphrodisiac and it is extensively used by local populations as part of traditional health care systems. The pygmies and bushmen of West African tropical zones certainly knew of the yohimbe power at an early date. Bantu-speaking tribe members were also aware of the faculty of this tree and they used decoctions made from its bark for marriage ceremonies and ritual orgies (Rätsch, 1997). Yohimbe is traditionally employed among other indications as a local anesthetic, sympatholytic, hypertensive drug in small doses, as well as hypotensive and hallucinogen in large doses, but the most common use remains as an aphrodisiac (Lewis and Elvin-Lewis, 1977; Oliver-Bever, 1986). In addition to its extensive indigenous use, the bark of yohimbe has been exported for many years to Europe, where the

‡ Most people think that dietary supplements and herbs are closely regulated to ensure that they are safe, effective, and truthfully advertised. Herbal supplements are considered foods, not drugs, by the US Food and Drug Administration (FDA) and, therefore, are not subject to the same testing, manufacturing, and labeling standards and regulations as drugs. Until 1994, the FDA had disallowed health claims of any kind on herbal supplements. The passage of the Federal Dietary Supplement and Health Education Act (DSHEA) in late 1994 started to reverse this trend and severely limited the FDA's ability to regulate these products. In January 2000, the FDA updated the laws governing the labeling of herbal supplements, so consumers now can see labels that explain how herbs can influence different actions in the body. However, herbal supplement labels still cannot say anything about treating specific medical conditions, because they are not subject to clinical trials or to the same manufacturing standards as prescription or traditional over-the-counter drugs.

drug is both prescribed in western medicine and sold in herbal markets. Its interest outside the African continent was first recorded in Germany, where yohimbine, the main alkaloid of the drug and also known as aphrodine, quebrachine or corynin, was isolated for the first time by Spiegel in 1896 (Morales, 2000). Recent advancements in the development of yohimbe-based remedies have led to an increased demand for the export market.

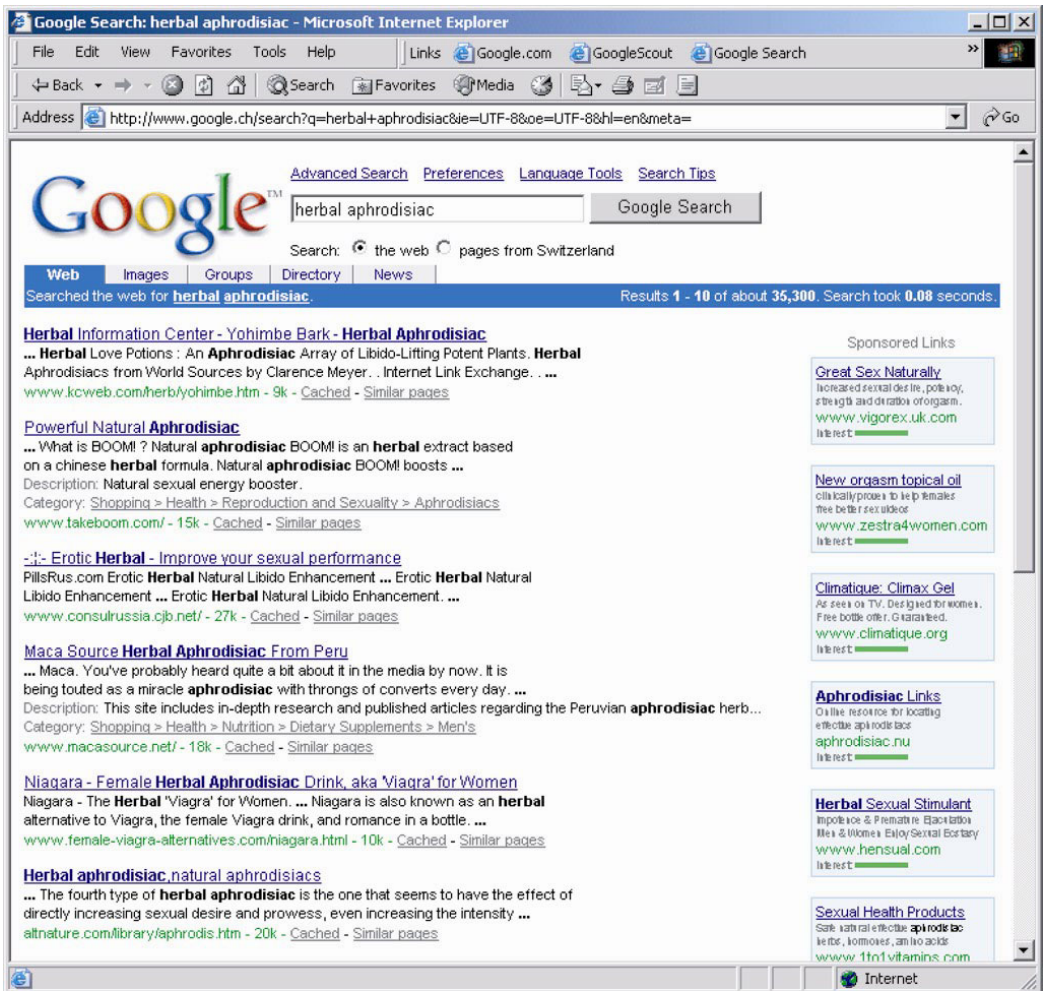


Figure 0-1

Results obtained in a search for aphrodisiacs on the Internet by a generic search engine.

Exploitation of *P. yohimbe* bark currently takes place in Cameroon and, to a lesser extent, Southeast Nigeria from wild populations (Sunderland *et al.*, 1999). Around 100 tons of bark are annually supplied by Plantecam (Cameroon), a subsidiary of Groupe Fournier, which is the exclusive supplier to Europe. Outside contractors, registered local businesses who have Forestry Department licenses to exploit medicinal plants, take advantage of all the *P. yohimbe* bark

supplied to Plantecam. Plant material from companies or individuals without valid licenses is not accepted by Plantecam; however the issue of licenses does not necessarily control the means of exploitation, and Plantecam themselves admit that "… 98% of the trees exploited are probably felled". The majority of *P. yohimbe* bark are collected by *BaKola* (pygmy) harvesters without any permits or authorization, carried to the roadside and illegally sold to contractors (Figure 0-2). Despite current levels of exploitation, the yohimbe tree presently grows abundantly and there does not seem to be a problem of regeneration. However, although the current regenerative capacity of the species is not yet compromised, the considerable over-exploitation in certain areas will ultimately affect the regenerative potential of *P. yohimbe* (Sunderland *et al.*, 1999).

Figure 0-2

Exploited bark of *P. yohimbe* in Southern Cameroon. Photo T. Sunderland.

1.2. Chemical components of *Pausinystalia yohimbe*

Authentic bark of yohimbe contains up to 5.9% of total alkaloids, depending on the age of the plant and the height at which the bark has been collected (Paris and Letouzey, 1960). During the rainy season, the alkaloid amount of the bark can increase by about 1% (Paris and Letouzey, 1960). The profile of alkaloids in the bark of yohimbe was reported as: yohimbine (17α-hydroxy-yohimban-16α-carboxylate, syn.: quebrachine), 0.8%; β-yohimbine (syn.: amsonine), 0.03%; and pseudoyohimbine (syn.: yohimbene), 0.04% (Le Hir *et al.*, 1953). Another study on two other bark samples gave the following results: yohimbine, 1.15 and 2.24%; α-yohimbine (syn.: corynanthidine, isoyohimbine, mesoyohimbine, and rauwolscine), 0.46 and 0.39%; pseudoyohimbine, 0.36 and 0.02%; ajmalicine (syn.: δ-yohimbine, raubasine, vinceine, and vincaine), 0.08 and 0.04%; and dihydrositsirikine, 0.12 and 0.39% (van der Meulen and van der

Kerk, 1964). Additional minor alkaloids have also been isolated: alloyohimbine (syn.: dihydroyohimbine), corynanthine (syn.: rauhimbine), corynantheine and dihydrocorynanthine (Kutney and Brown, 1966; Le Hir *et al.*, 1953). Some structures of these molecules are illustrated in Figure 0-3. The references for the synonyms are given in the book of M. Hesse (Hesse, 1964).

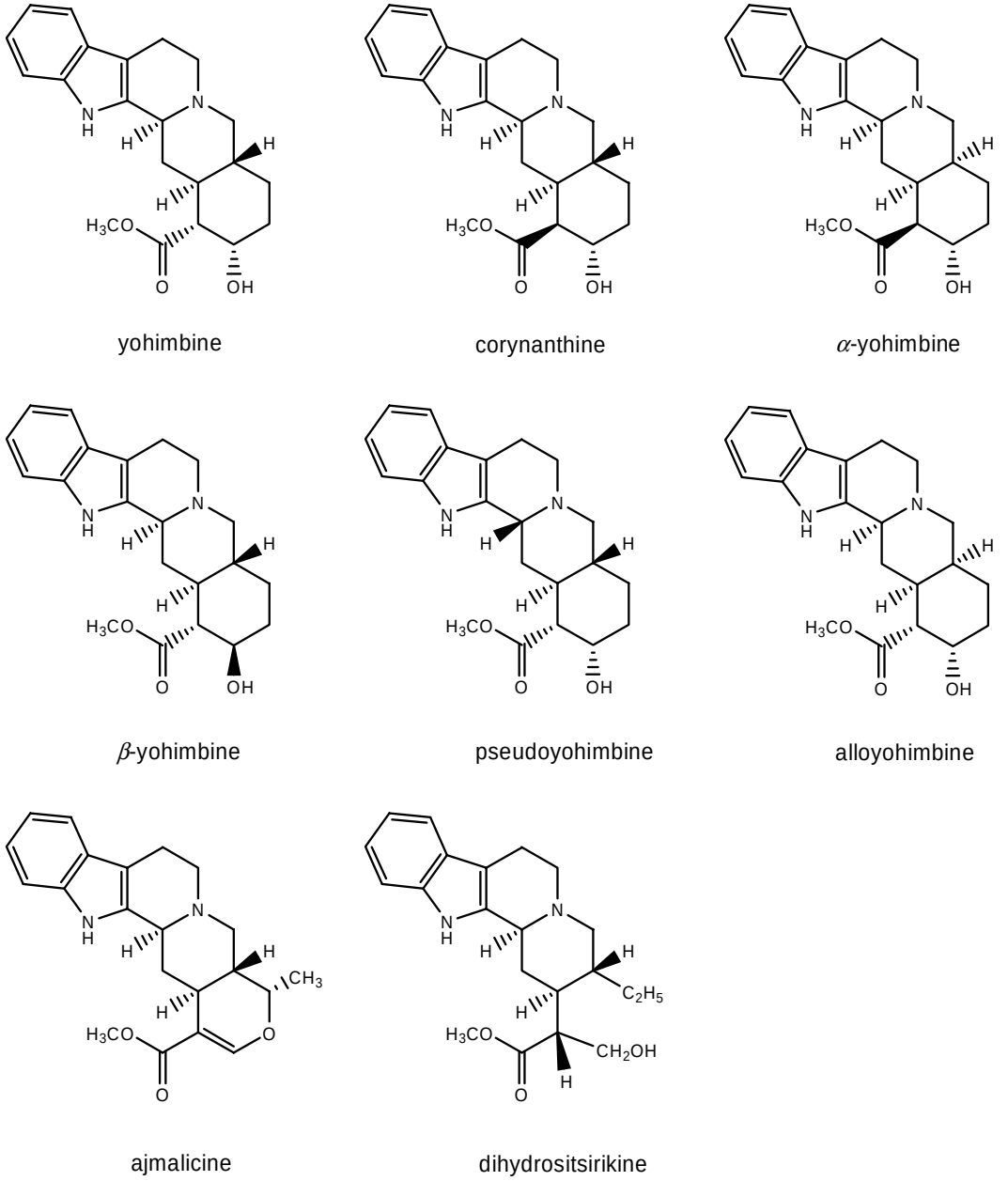


Figure 0-3

Structures of principal indole alkaloids of *P. yohimbe*.

1.3. Pharmacological effects of yohimbine

From a pharmacological point of view, yohimbine is a competitive antagonist selective for the α_2-adrenergic receptor (Murburg *et al.*, 1991). Its α_2-antagonist activity is 20 to 500 times stronger than its α_1-activity, and also depends on the specific tissue (Steers *et al.*, 1984). The sympathetic outflow increases as a result of the blockade of the presynaptic α_2-adrenergic receptors as well as by potentialization of the release of norepinephrine from nerve endings (Figure 0-4). This leads to an activation of α_1 and β_1 receptors in the heart and in the peripheral vasculature with a consequent rise in blood pressure (Goldberg *et al.*, 1984). On the other hand, blockade of the postsynaptic α_2-adrenergic receptors induces an inhibition of the sympathomimetic reactions. Yohimbine is considered a "dirty drug" because, in addition to its effects in the sympathetic system it exhibits other important actions, including dopamine receptor antagonism, monoamine oxidase (MAO) and cholinesterase inhibition, and 5-hydroxytryptamine receptor antagonist and agonist activity (Goldberg and Robertson, 1983). The drug is metabolized with rapid plasma clearance, exhibiting a biological t½ of 36 minutes, and is not eliminated in the urine (Owen *et al.*, 1985; Owen *et al.*, 1987).

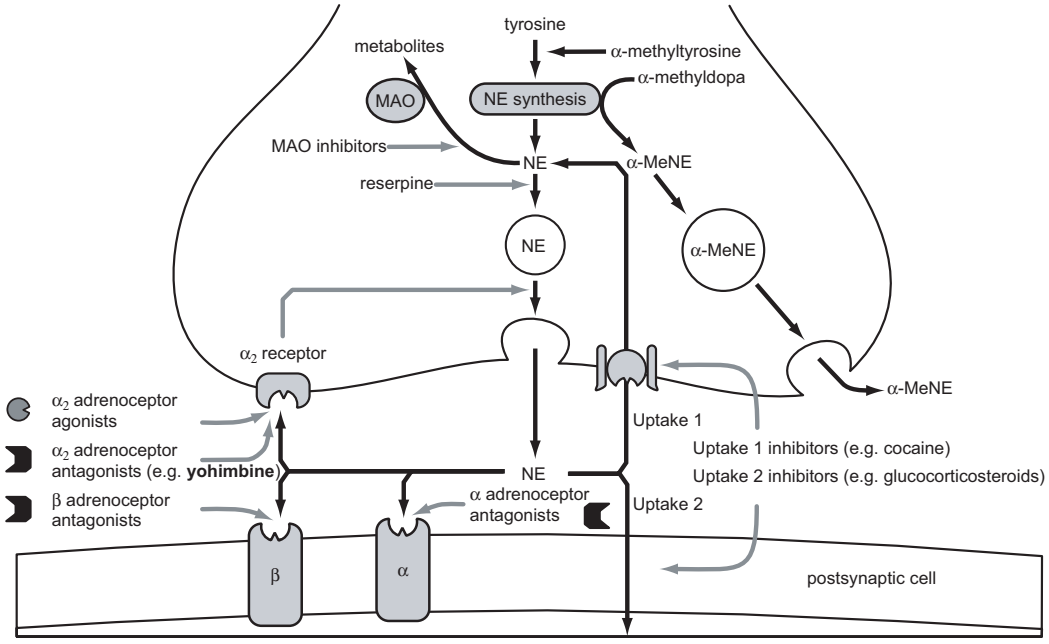


Figure 0-4

Drug actions on the sympathetic nervous system. Drugs may affect synthesis, storage, release, uptake, and receptors. Yohimbine blocks the presynaptic α_2-adrenergic receptors. (MAO, monoamine oxidase; α-MeNE, α-methylnorepinephrine; NE, norepinephrine)

As mentioned in chapter 1.1, yohimbine has been used in the past as an aphrodisiac and erectogenic drug. However, even if this drug is pharmacologically characterized, its efficacy in the treatment of sexual dysfunction has never clearly been demonstrated. The positive effect of yohimbine on sexual activity in male rats is well defined (Clark *et al.*, 1984, 1985), but unfortunately the situation is not nearly as clear for man (Morales, 2000). According to the literature (Feuerstein *et al.*, 1985), the activity of yohimbine in the peripheral and central nervous system should not be restricted to the noradrenergic system but also extended to the serotonergic system. A possible mechanism for the improvement in erectile function of patients treated with yohimbine was established after the identification and characterization of functional α_2-adrenergic receptors in human corpus cavernosum (Traish *et al.*, 1997). On the other hand, according to recent data (Sonda *et al.*, 1990) the central nervous system activity of yohimbine appears to be predominant for a sexual response. From a therapeutic point of view, it was suggested that this duality may in fact be beneficial for yohimbine activity even though the use of non-selective drugs is generally precluded (Morales, 2000). Until now, only a dozen controlled studies (Hollander and McCarley, 1992; Jacobsen, 1992; Kunelius *et al.*, 1997; Morales *et al.*, 1982; Price and Grunhaus, 1990; Riley, 1994; Rowland *et al.*, 1997; Sonda *et al.*, 1990; Susset *et al.*, 1989; Vickers *et al.*, 1993; Vogt *et al.*, 1997) have been reported and some of them are statistically insufficient for a definitive conclusion. In all these studies, administration of a daily dose of 18-42 mg of yohimbine often divided in three single doses, demonstrated only modest effects on psychogenic erectile dysfunction (Reid *et al.*, 1987) and a marginal efficacy in selected cases of organic impotence (Morales *et al.*, 1987). A 2- to 3-weeks latency between onset of daily yohimbine administration and this light erectile function improvement was observed. Some authors (Teloken *et al.*, 1998) studied the effects of a large daily single dose (100mg) of yohimbine compared to placebo, and concluded that the drug "promotes no improvement in patients with organic erectile dysfunction". The American Urological Association guidelines on treatment of organic erectile dysfunction recently stated that "the outcome data for yohimbine clearly indicate a marked placebo efficiency" and that "based on the data to date, yohimbine does not appear to be effective for organic erectile dysfunction and, thus, it should not to be recommended as treatment for the standard patient" (Montague *et al.*, 1996). Besides these results, two different meta-analyses (Carey and Johnson, 1996; Ernst and Pittler, 1998) demonstrated that yohimbine is superior than placebo in the treatment of erectile dysfunction.

1.4. Side effects and contraindications of yohimbine

Despite studies which show that yohimbine is generally well tolerated with few and benign side effects (Morales *et al.*, 1987; Riley, 1994; Sonda *et al.*, 1990; Susset *et al.*, 1989; Vogt *et al.*, 1997), other reports illustrate its potential risks on certain individuals. Common adverse effects of yohimbine include elevated systolic blood pressure and heart rate as well as anxiety, irritability and tremor, headache, mild antidiuretic activity (stimulation of antidiuretic hormone release), nausea, increased perspiration, salivation, lacrimation, and papillary dilatation (Grossman *et al.*, 1993; Gurguis and Uhde, 1990; Lacomblez *et al.*, 1989; Onrot *et al.*, 1987; Price *et al.*, 1984; Teloken *et al.*, 1998). Yohimbine was also reported to induce a generalized erythrodermic skin eruption, progressive renal failure and lupus-like syndrome (Sandler and Aronson, 1993). Furthermore, a daily dose of 15 mg can provoke bronchospasms (Landis and Shore, 1989). Treatment with yohimbine is contraindicated in patients taking psychotropic medications and tyramine-containing food because of its potential effects on cholinergic and adrenergic activities (Arky, 1998). Description of a hypertensive crisis due to an herbal treatment of impotence suggested that these medications are a potential cause of toxicity (Ruck *et al.*, 1999). The German Commission E monograph recommended that the *P. yohimbe* bark should not be taken by patients with renal disease, noting increases in blood pressure.

With regard to these data, detection and quantification of yohimbine in the commercialized products have to be considered imperative, in order to evaluate the potential risks of aphrodisiac products on consumer health.

1.5. Previous detection and quantification methods

Thin-layer chromatographic methods for identifying yohimbine in authentic yohimbe bark have been reported in the literature (Wagner and Bladt, 1996). More sensitive methods using HPLC coupled with UV spectrophotometry, amperometric or fluorescence detection have been employed to determine and quantify this compound (Chiba and Isii, 1990; Diquet *et al.*, 1984; Goldberg *et al.*, 1984; Le Verge *et al.*, 1992; Owen *et al.*, 1985). A more selective analysis of yohimbine using the LC-MS-MS technique has been recently published (Reimer *et al.*, 1993). All these methods have been employed to analyze clinical samples from persons using mono-substance medication (yohimbine), but they were not sensitive and selective enough for the investigation of multibotanical products (Betz *et al.*, 1995). A GC method to analyze dietary supplements containing complex plant mixtures including yohimbe bark was thus developed. A nitrogen phosphorous detector (NPD) was used to quantify total alkaloids and identification of

yohimbine was established by comparison of GC-EI-MS spectra with the reference mass spectrum obtained for the yohimbine standard (Betz *et al.*, 1995).

The present study describes a rapid qualitative and quantitative method for the analysis of yohimbine in commercialized aphrodisiacs by HPLC-UV-MS with atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). The optimized experimental conditions allowed a considerable reduction of the time of analysis and simplified the preparation procedure. The precision and reproducibility of the MS analysis was improved by adding deuterium labeled yohimbine as internal standard (Auriola *et al.*, 1991a, b). The HPLC-MS method proved to be more sensitive when compared to HPLC-UV. The selectivity of the MS detection has been improved by a MS^n analysis of the yohimbine chromatogram peaks.

2. Results and Discussion

2.1. General procedures

Aphrodisiac products were purchased from different sources found on the Internet (see chapter VI.1.1). Figure 0-5 illustrates an example of herbal aphrodisiacs obtained by internet resellers and analyzed in this study.

Figure 0-5

Aphrodisiac products obtained from Internet retailers and analyzed for their yohimbine content.

Extractions. The extraction procedures for authentic yohimbe bark and aphrodisiac products are reported in chapter VI.1.1.

Sample preparation. The residues of extractions were dissolved in 50.0 mL of an acetonitrile (2 mM Et_3N): water (50:50) solution containing codeine (1 mM) for UV detection and deuterium-labeled yohimbine (0.4 mM) for MS detection as internal standards. The deuterium-labeled yohimbine ([methyl-^{2}H]-labeled yohimbine) was obtained by esterification of yohimbinic acid with [^{2}H]-methanol in acidic conditions (see chapter VI.1.12).

Preparation of standard solutions. Pure reference substance yohimbine HCl was dissolved in an acetonitrile (2 mM Et_3N): water (50:50) solution containing codeine (1 mM) and deuterium-labeled yohimbine (0.4 mM) as internal standards in a 100-mL volumetric flask. Dilutions were prepared from this solution to establish a calibration curve in the range of 47.4-1896.5 ng on-column (n=7). A volume of 10 µL was injected.

Chromatography. HPLC instrumentation and conditions are reported in chapter VI.2.2. Separation was performed on a Nucleosil 100-5 C_{18} AB column (125 x 2 mm i.d., 5 µm; Macherey-Nagel, Düren, Germany) equipped with a pre-column of the same material (8 x 3 mm i.d., 5 μm) using a water (2 mM Et_3N) : acetonitrile (2 mM Et_3N) gradient of 80:20 to 70:30 in 2 min, followed by an isocratic elution of 70:30 for 6 min, a gradient elution of 70:30 to 55:45 in 6 min and to 0:100 in 6 min. The total analysis time was 35 minutes, including column washing and stabilization. The flow-rate was set to 0.3 mL/min and detection to 280 nm.

LC-API-MS. LC-APCI-MS and LC-ESI-MS instrumentation and conditions are reported in chapter VI.2.3. MS^n experiments were performed by programming dependent scan events. The first event was a full MS scan (MS^1); during the second event the main ion recorded was isolated and selectively fragmented in the ion trap (MS^2), whilst the third event (MS^3) represented the isolation and selective fragmentation of the most important ions recorded in the MS^2. The collision energy was set to 30 eV. To confirm the fragmentation way of yohimbine and its epimers, a 10 µg/mL solution of the corresponding standards was examined by flow injection analysis (FIA) using the APCI-MS^n conditions described for the LC-APCI-MS analyses. The standard solution injection flow was 5 µL/min and was diluted before the APCI interface by addition of a 40:60 acetonitrile (2 mM Et_3N):water (2 mM Et_3N) solution performed with a HP-1100 (Hewlet Packard, Palo Alto, CA, USA) binary pump (0.2 mL/min) by means of a T junction (Waters, Milford, MA, USA).

2.2. Validation requirements

The validation of the analytical test method was carried out to ensure that the analytical methodology was accurate, specific, reproducible and robust over the specified range that the

analyte was analyzed. Analytical method validation has received considerable attention in the literature and regulatory agencies but unfortunately, there is no single source of final guidelines on it (Shabir, 2003). In this study, guidelines from the International Conference on Harmonization (ICH, 1995, 1997) were used to perform the validation.

Before undertaking the task of method validation, some preliminary work was performed (*i.e.*, analytical equipment qualification, stability of analytical solutions and system suitability test). The analytical system itself was evaluated for its adequate design, maintenance, calibration, and testing. Before performing any validation experiments, the installation, operational and performance qualification was estimated. Afterwards, the HPLC system was tested for its suitability, which is the capability of providing data of acceptable quality. In addition, to generate reproducible and reliable results, the stability of sample solutions, standards, reagents and mobile phases was determined.

According to the ICH, the characteristics used to "quantify" the performance of the analytical method included the following:

Accuracy. Accuracy is the measure of the exactness of an analytical method, or the closeness of agreement between the value which is accepted either as a conventional, true value or an accepted reference value and the value found. For the assay of the drug products, accuracy was evaluated by analyzing mixtures spiked with known quantities of components (technique of standard additions). In general, this approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte. Accuracy criteria for the assay method were that the mean recovery would be 100 ± 2% at each concentration over the range of 70-130% of the target concentration. To document accuracy, the ICH guideline on methodology recommends collecting data from a minimum of nine determinations over a minimum of three concentration levels covering the specified range (*i.e.*, three concentrations, three replicates each).

Precision. Precision is the measure of the degree of repeatability of an analytical method under normal operation and is usually expressed as the percent relative standard deviation (RSD) for a statistically significant number of samples. The precision includes repeatability, intermediate precision and reproducibility. The repeatability is the results of the method operating over a short time interval under the same conditions (intra-assay precision). This implies the use of the same operator, equipment and reagents. It was calculated from nine determinations covering the specified range of the procedure (*i.e.*, three levels, repeated three times each). The intermediate precision is the measurement of day-to-day method variations due to random events within the same laboratory. The reproducibility is the measure of the precision of the method when changing all of the possible variables, *i.e.* different operators, reagents,

instruments, times and laboratories. It is normally acceptable if it is no more than two or three times greater than the repeatability.

Specificity. The specificity is the ability of the method to measure one species of analyte in the presence of other elements or compounds. To measure the specificity of the HPLC methods, the analyte peak purity and resolution from the nearest eluting peak was calculated. Once acceptable resolution was obtained for the analyte, the chromatographic parameters, such as column type, solvent system, flow-rate and detection mode, were considered set.

Limit of detection. The limit of detection is the indication of the minimum analyte concentration in a sample that can be detected, not quantitated. It is a test that specifies whether or not an analyte is above or below a certain value. It was measured as a concentration at a 3:1 signal-to noise ratio and an appropriate number of samples were analyzed at this limit to validate the level.

Limit of quantitation. The limit of quantitation is the indication of the minimum analyte concentration in a sample that can be measured with acceptable precision and accuracy under the stated operational conditions of the method. The limit of quantitation was set to a signal-to-noise ratio of 10:1. Afterwards, the precision and the accuracy were determined at this concentration to confirm the validation of the method.

Linearity and range. The linearity identifies the range of concentrations over which the method response is proportional to the amount of analyte. The straight-line over the range does not necessarily extrapolate to the origin but would normally be expected to do so. A correlation coefficient of >0.999 is generally considered as evidence of acceptable fit of data to the regression line. Range is the interval between the upper and lower levels of analyte (inclusive) that have been demonstrated to be determined with precision, accuracy and linearity using the method as written. The minimum recommended range for assay is from 80-120% of the target concentration.

Robustness. Robustness is the capacity of a method to remain unaffected by small deliberate variations in method parameters. The robustness was considered early in the method development and was evaluated by varying column temperature (±1 to 5°C), percent organic solvent (± 2% acetonitrile content in mobile-phase), pH of buffer in mobile phase (up to ± 0.5 pH units), flow-rate (± 0.1 mL/min), etc. These changes were within the limits that produce acceptable chromatography and were incorporated in the method procedure.

ICH recognizes that is it not always necessary to evaluate every analytical performance parameter and indicates which one is needed depending on the type of method and its intended use (Table 0-1).

Table 0-1

ICH validation characteristics.

Analytical performance characteristics	Identification	Impurity testing		Assay
		Quantitative	Limit tests	
Accuracy	No	Yes	No	Yes
Precision				
Repeatability	No	Yes	No	Yes
Intermediate precision	No	Yes	No	Yes
Specificity	Yes	Yes	Yes	Yes
Limit of detection	No	No	Yes	No
Limit of quantitation	No	Yes	No	No
Linearity	No	Yes	No	Yes
Range	No	Yes	No	Yes

2.3. Method development

Extraction procedures. A number of extraction methods have been described, relating almost entirely to subsequent analysis of the extract by HPLC, which is the predominant final analytical method. The extraction method reported by Betz *et al.* (1995) was chosen because of its application to commercial yohimbe products. In general, the extraction was carried out by grinding the authentic bark or aphrodisiac products, making them alkaline with 30% NH_4OH and extracting them in $CHCl_3$. Extraction procedures varied slightly from those proposed by Betz *et al.* (1995) and therefore had to be validated. Figure 0-6 graphically shows the decreasing of yohimbine in authentic yohimbe bark with the increasing number of extractions. The measurements were made in triplicate and they showed that after the fourth extraction the quantity of yohimbine remaining was negligible. For commercial aphrodisiacs, the extraction procedures were adapted to the specific dosage form.

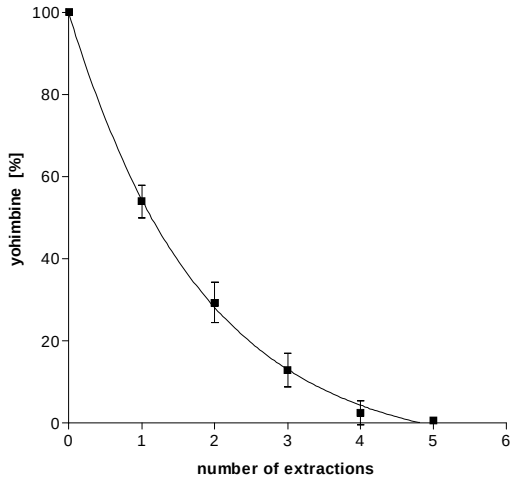
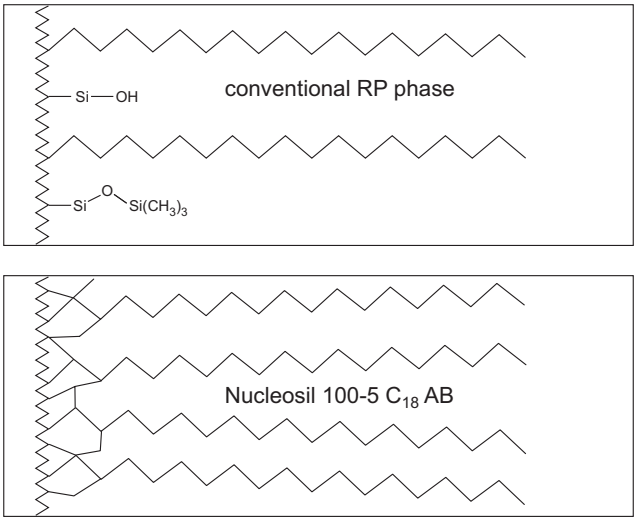


Figure 0-6

Graph representing the quantity of yohimbine in authentic yohimbe bark after a certain number of extractions (solvent: alkaline $CHCl_3$; extraction time: 1.5 hr; temperature: 23°C; replicates: n = 3).

HPLC-API-MS. In order to analyze the alkaloid extract of multibotanical samples, a LC-MS method was optimized through the development of a fast and robust LC chromatographic separation and detection of yohimbine with two different ionization interfaces. The reversed-phase chromatographic separation of the alkaloid extract was restricted by the compatibility with the LC-MS ionization conditions. The basic properties of alkaloids lead indeed to protonated molecules under acidic and neutral conditions, increasing their polarity and decreasing their affinity to the lipophilic-stationary phase. Moreover charged molecules were responsible for hydrogen bonding and ionic interactions with deprotonated silanol groups leading to additional retention and peak tailing. Tailing is extremely undesirable when separating complex mixtures of compounds due to loss of resolution, and also due to the effects it has on quantitative results. In fact, integrators may not be able to establish the end of a tailing peak with sufficient precision and accuracy. An answer to those problems was to work with a basic solvent system or use acid buffers with ion pair reagents which have high affinities for protonated alkaloids and RP phase. A highly efficient HPLC method for the separation of indole alkaloids (including yohimbine and its epimers) has already been developed (Gerasimenko *et al.*, 2001), but the use of a high concentration of phosphate buffer (39 mM) and hexanesulphonic acid (2.5 mM) as ion pair reagent was not compatible with the LC-APCI-MS and LC-ESI-MS interfaces. The use of triethylamine (Et_3N) (2 mM) allowed a good separation of the alkaloids on reversed phase and this buffer did not appear to interfere with yohimbine ionization under experimental LC-MS conditions. Due to their limited stability,

conventional RP stationary phases were not recommended to be run with mobile phases at pH > 8 for extended periods of time. Cleavage of the siloxane bonding by hydrolysis, or dissolution of the silica would rapidly lead to a considerable loss in column performance. Moreover, older "classical" RP supports, which are made from lower purity silica than more recent phases, seemed to give worse separation results. It is possible that the poorer performance may be attributed to their high metal content, *i.e.* iron and aluminum which can become incorporated into silica structure, increasing the acidity of neighboring silanol groups. To solve this problem, a new base-deactivated Nucleosil® 100-5 C_{18} AB column was employed. The polymer-coated octadecyl packing material features an outstanding shielding of the silica matrix due to its chemically cross-linked C_{18} modification and is inert towards basic substances. The carbon content of 25% is considerably higher than for monomerically coated standard C_{18} phases (Figure 0-7). This special surface bonding technology of Nucleosil® 100-5 C_{18} AB increases the stability to hydrolysis in alkaline media when compared to conventional RP phases and allows the use of enlarged pH range from 1 to 11. Moreover, this special phase has a particularly large steric selectivity.



Surface of silica contains active groups that can cause unwanted interactions with the analytes, even after derivatization and encapping.

Base deactivated octadecyl-phase with polymeric coating reduce silanol interactions.

Figure 0-7

Representation of a conventional RP phase and a base deactivated RP phase.

The response in all LC-MS techniques is highly dependent upon pH. Knowing the pKa value of yohimbine (7.26 at 23°C) allowed us to predict its state of ionization as a function of pH. LC-MS ionization of yohimbine was compared using two different interfaces -APCI and ESI. APCI requires the sample to be vaporized, favoring vaporization of neutral species in a liquid phase because of their higher volatility in comparison with charged species. In the present analysis,

adjusting the pH to a basic value with Et_3N aided yohimbine and other alkaloids in their volatilization. With APCI, ions were observed in both positive and negative modes, but the negative ion mode failed to give a stable and reproducible MS response. Furthermore, peak intensity observed in the negative mode with APCI ionization was weak. On the other hand, adjusting the pH to favor charged molecules can be helpful for desorption techniques such as ESI. The desorption process is thus favored for charged species in comparison with neutral species, due to repulsion with other like-charged ions. Figure 0-8 illustrates the regions that favor desorption in contrast to vaporization at different pH values.

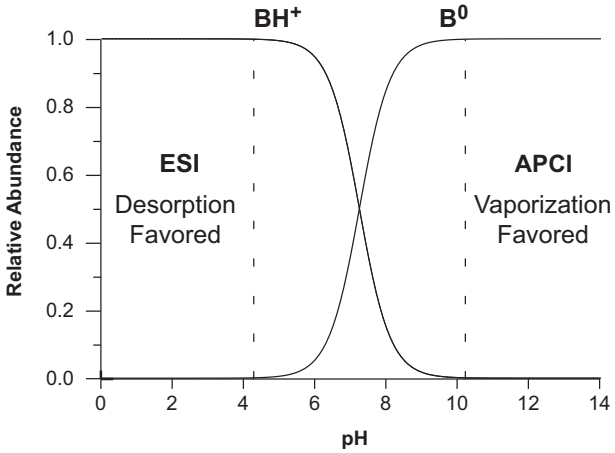


Figure 0-8

Favored pH regions for ESI and APCI: desorption and vaporization. Relative abundance of yohimbine (B^0) and its conjugate acid (BH^+) at various pH values. Yohimbine pKa: 7.23 at 23°C.

In our case, the ideal pH for LC separation (>10.23 at 23°C; 3 units more than yohimbine pK_a) was not optimal for an ESI interface. A post-column removal of buffer or neutralization could be a solution to this problem but unfortunately it was not suitable for quantitative analysis, because it affected the precision. Surprisingly, ions measured in the positive mode were found to give a stable, reproducible and highly sensitive MS response. This observation was probably due to the redox reactions of water that take place in the metal capillary of ESI in the positive ionization mode. A recent study (Van Berkel *et al.*, 1997) reported that the pH of the spraying solution may decrease by at least 4 pH units with the ESI interface due to electrolytic oxidation of water in the positive mode. The pH changes were promoted when using a metal spray capillary and increased as the flow rate decreased and/or the ESI current increased.

The LC method with Et_3N buffer was finally retained using both APCI-MS and ESI-MS techniques for qualitative and quantitative analysis of the different extracts. The pH minimum

was set to 10.23 at 23°C in order to have yohimbine quantitatively in its basic form. The two API were performed in the positive ion mode. In order to improve the quantitative analysis precision, codeine and isotopically-labeled yohimbine were added simultaneously as internal standards during preparation of the sample. Quantitation is based on the response ratio of the compound of interest (yohimbine) to the internal standard (codeine or yohimbine-d_3) versus the response ratio of a similar preparation of the reference standard. The internal standard method is appropriate for complex sample preparation procedures (*e.g.*, multiple extractions), low concentrations of sample (sensitivity being an issue), and wide range of concentrations expected in the sample for analysis. Codeine was chosen as internal standard because of its chemical character and the similarity of its UV properties to yohimbine: it possesses a chromophore that shows a maximum of absorbance at 280 nm. In addition, using the LC conditions described before, the peak of codeine was in general well separated from peaks of other substances. The inconvenience of this UV active internal standard was the lack of selectivity, which in multibotanical products could be determinant. The use of deuterium-labeled yohimbine as internal standard for the MS analysis appeared to be a more elegant method to avoid variations in sample preparation, injection and in particular ionization parameters. Moreover, the MS response of yohimbine began to fluctuate during a sequence with more than 50 injections, especially with the ESI interface.

Under the optimized conditions, qualitative analysis of the chloroform extract of authentic yohimbe bark was performed in the full scan mode (*m/z* 100-800) to estimate its content and determine the specificity of the method. Figure 0-9 shows MS and UV chromatograms of this extract with respective spectra of the target analyte.

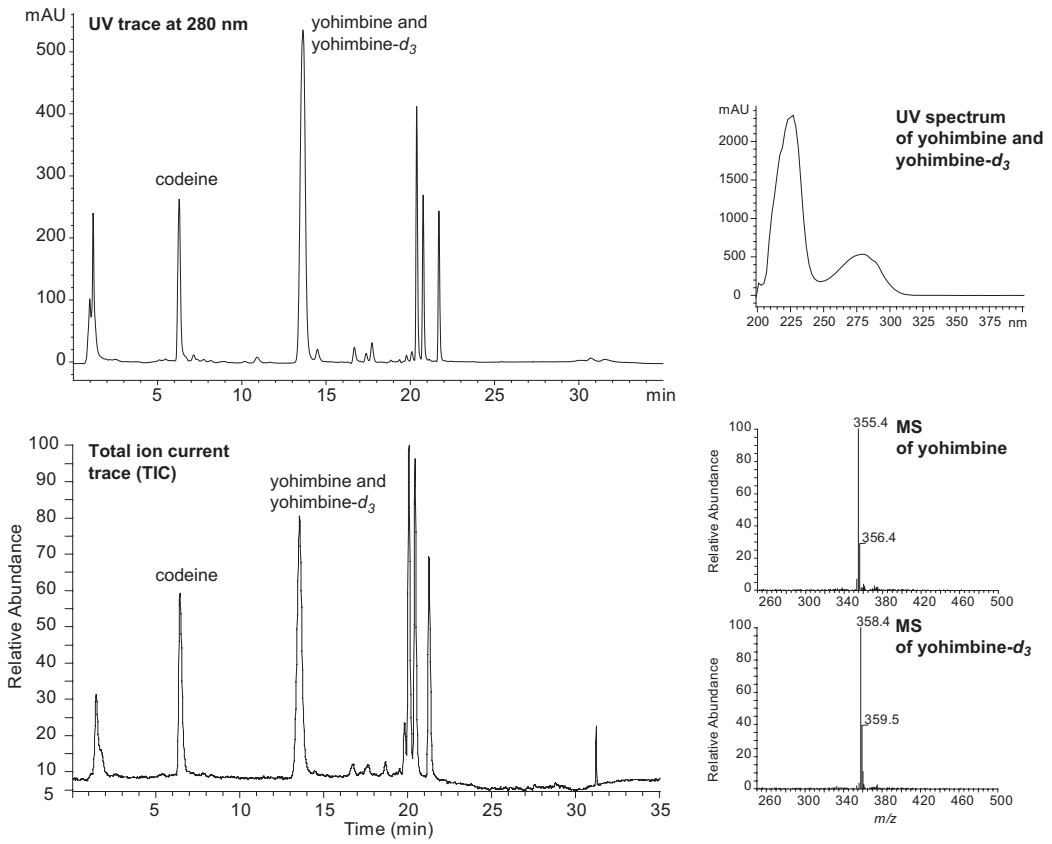


Figure 0-9

Representative LC-UV-APCI-MS analysis of the authentic yohimbe bark extract containing the internal standards codeine and yohimbine-d_3 (1277 ng on column). Column: Nucleosil 100-5 C18 AB (125 x 2 mm i.d., 5 μm) with a pre-column of the same material (8 x 3 mm i.d., 5 μm). Eluents: water (A) and acetonitrile (B) with 2 mM Et3N. Gradient: 20-30% B in 2 min, isocratic elution at 30% B for 6 min, 30-45% B in 6 min and 45-100% B from 14-20 min were applied. Flow rate: 0.3 mL/min. UV detection at 280 nm. MS conditions: capillary temperature: 150°C; vaporizer temperature: 360°C; corona needle current: 6.0 μA; sheath gas (nitrogen) pressure: 90 psi; in-source CID: –15 eV.

In the LC-UV-MS analysis, yohimbine and its deuterium-labeled analogue were observed at a retention time of 13.63 min and their UV spectra showed a maximum of absorbance at 280 nm. Codeine gave the same maximum of absorbance and a retention time of 6.28 min. The MS spectrum of the yohimbine peak showed two protonated ions $[M+H]^+$ at *m/z* 355.4 and 358.4 corresponding to protonated yohimbine and its protonated trideuterated analogue, respectively. Significantly, a protonated ion $[M+H]^+$ was also seen for both molecules in the MS spectrum at *m/z* 356.4 and 359.5, related to their respective isotopes.

The selectivity of the developed method was tested by analyzing three chloroform extracts of aphrodisiac products by LC-UV-MS^n. The fragmentation patterns of the yohimbine and codeine peaks in these extracts were identical to those of the respective pure compounds, indicating the

absence of other molecules having the same retention times and UV spectra. Figure 0-10 illustrates the UV and MS chromatograms of one of these extracts (aphrodisiac product 9; see Table VI-1) and the fragmentation pattern of the yohimbine peak.

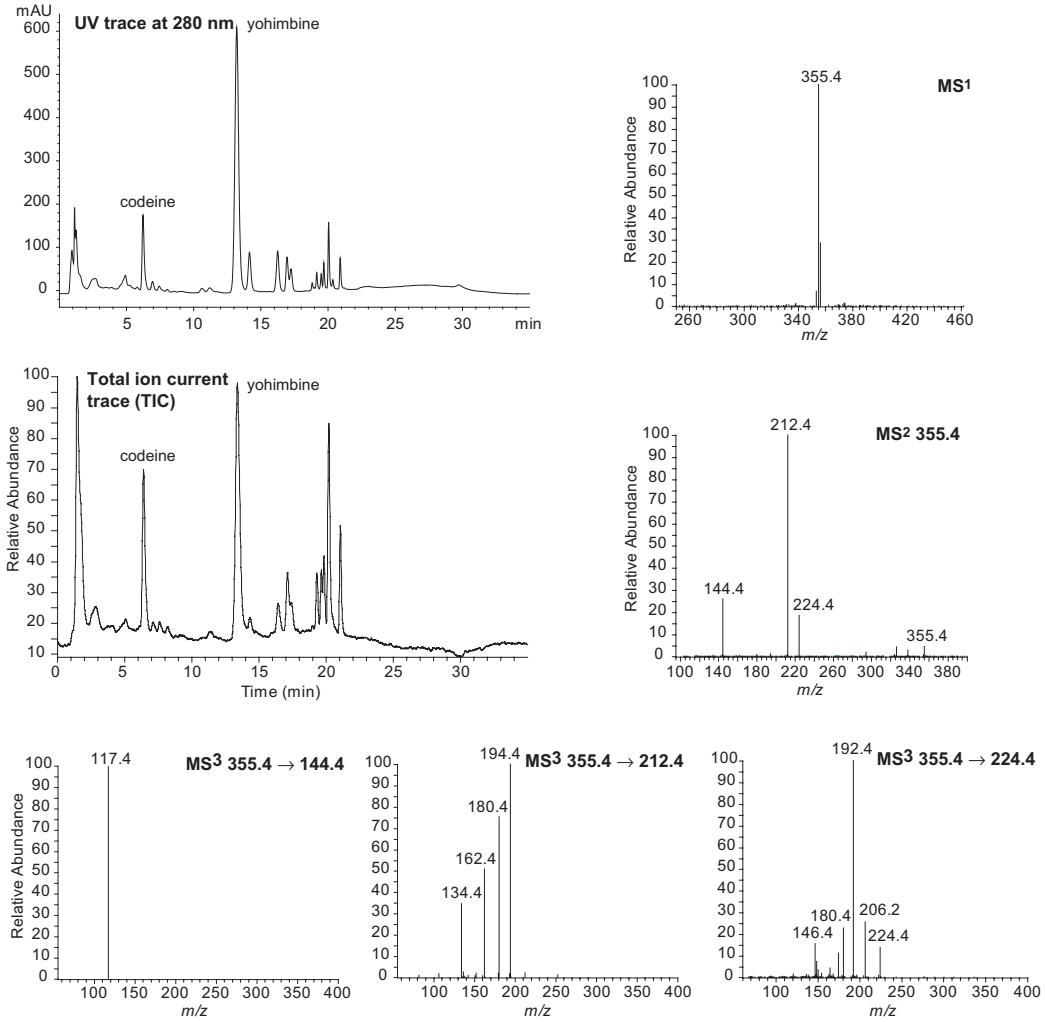


Figure 0-10

LC-UV-APCI-MS^3 analysis of the extract of aphrodisiac product **9**. (Same conditions as described in legend for Figure 0-9. The collision energy was set to 30%).

2.4. Detection and quantification

In order to increase the sensitivity of the developed method, qualitative and quantitative determination of yohimbine was performed by single ion monitoring (SIM) detection of $[M+H]^+$ ions at *m/z* 355.4 and 358.4 with a spectral window of ± 0.6 Da (Figure 0-11).

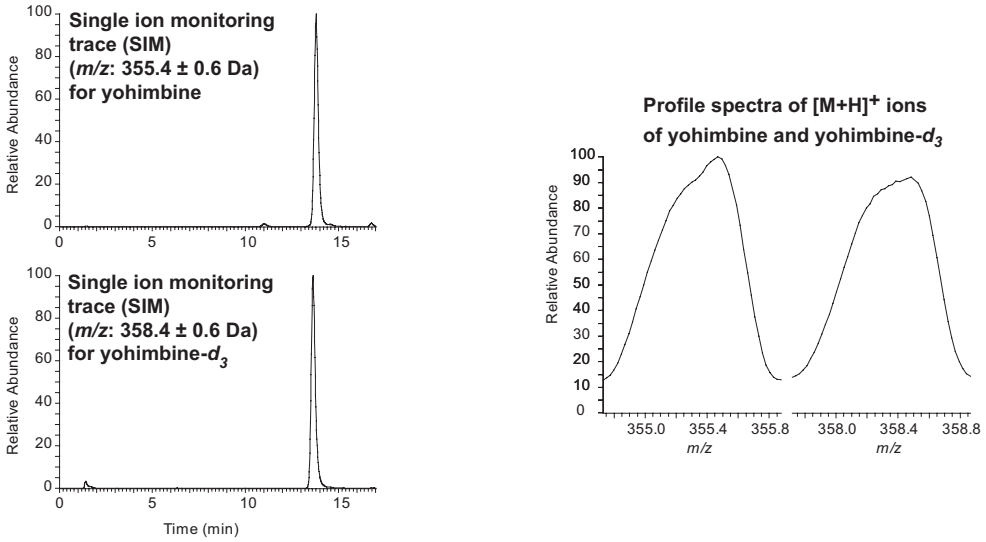


Figure 0-11

Single ion monitoring (SIM) detection of yohimbine and its trideuterated analogue. (Same conditions as described in legend for Figure 0-9. Detection at *m/z* 355.4 and 358.4 with a spectral window of ± 0.6 Da).

Consistent parameters of quantification were determined for UV and MS methods (Table 0-2). The calibration curves were established by injecting in triplicate seven samples of pure yohimbine in the 47.4-1896.5 ng (4.74-189.65 µg/mL) range. Linear regression was determined by calculating the ratio of the areas of yohimbine and its internal standard as a function of concentration. Excellent correlation coefficients were obtained by UV ($r^2 = 0.9997$), APCI ($r^2 > 0.9997$) and ESI ($r^2 > 0.9998$) methods. The detection limits for the qualitative analysis were evaluated using a signal-to-noise ratio, by comparing the measured signals of pure standard solutions of known concentrations with those of blank samples. A signal-to-noise ratio of 3:1 was considered acceptable and gave detection limits of 0.6 ng on column (60 ng/mL) for UV detection and 0.03 ng on column (3 ng/mL) for API techniques. The quantitation limits were also calculated using the same approach but considering a typical signal-to noise ratio of 10:1. The minimum concentration at which the analyte could be quantified was established at 2.0 ng on column (200 ng/mL) for UV detection and 0.1 ng on column (10 ng/mL) for API methods.

In order to estimate the precision of the methods, the repeatability was evaluated by successively injecting 15 standards with a concentration covering the linearity range (3 concentrations, 5 replicates each). The RSD showed a good repeatability for each technique. The intermediate precision was measured as for the repeatability but at different days and gave good precision results (RSD < 2%). Accuracy was established after determination of precision, linearity and specificity, and performed across the specified range. It was measured as the percent of analyte recovery, after spiking authentic yohimbe bark and products with 70, 100 and 130% of yohimbine (three concentration levels, three replicates each) before the extraction procedure. Recovery of target analyte from the authentic yohimbe bark was excellent (Table 0-2).

Table 0-2

Validation results of HPLC-UV, HPLC-APCI-MS and HPLC-ESI-MS methods for the detection and quantitative determination of yohimbine in aphrodisiac products.

	LC- UV	LC-APCI-MS	LC-ESI-MS
Accuracy (recovery 70-130%; 3 levels, n = 3)	99.5% ± 0.92%	102.7% ± 2.10%	103.1% ± 3.12%
Precision			
Repeatability (3 levels, n = 5)	91.8 ng (0.85%) 950.0 ng (0.47%) 1882.3 ng (0.35%)	89.3 ng (2.63%) 961.2 ng (0.45%) 1886.4 ng (0.74%)	88.3 ng (0.54%) 966.1 ng (1.26%) 1906.4 ng (0.20%)
Intermediate precision (3 levels, n = 5)	91.7 ng (0.19%) 949.9 ng (0.56%) 1883.0 ng (0.41%)	90.4 ng (1.01%) 958.7 ng (0.39%) 1885.1 ng (0.59%)	90.0 ng (0.74%) 953.8 ng (0.66%) 1899.5 ng (0.34%)
Specificity	YES	YES	YES
Limit of detection[a]	0.6 ng on column (60 ng/mL)	0.03 ng on column (3 ng/mL)	0.03 ng on column (3 ng/mL)
Limit of quantitation[a]	2.0 ng on column (200 ng/mL)	0.1 ng on column (10 ng/mL)	0.1 ng on column (10 ng/mL)
Linearity (7 levels, n = 3)			
Slope:	0.001240	0.0008131	0.0007787
y-intercept :	1.96	0.02896	0.01018
r^2:	0.9997	0.9997	0.9999
Range	47.4-3793.0 ng on column (4.74-379.30 µg/mL)	47.4-1896.5 ng on column (4.74-189.65 µg/mL)	47.4-1896.5 ng on column (4.74-189.65 µg/mL)

[a] Determination of limits based on a signal-to-noise approach.

Recoveries of yohimbine from the different matrices of aphrodisiac products varied with galenical forms. They were between 94.5 and 81.4% for gelatin capsules, 99.2 and 80.7% for tablets, 98.3 and 85.9% for liquid products, and 97.3 and 96.1% for powders or solids. This fluctuation of recoveries may have been due to the irreversible absorption of yohimbine by the matrix of different preparations. Herbal aphrodisiacs were in most cases multibotanical preparations containing a multitude of substances which could interact with each other. Furthermore, in many of these preparations amino acids (*i.e.*, L-arginine), hormones (*e.g.*, DHEA), vitamins and minerals were added which could bind to alkaloids. In addition, use of excipients such as fillers, binders, lubricants, colorants and aromas could be the cause of physical interactions with yohimbine. In order to increase these recovery results, different extraction procedures could be adopted for each commercial product, but this would definitely complicate and lengthen the entire quantification method. Finally, a single extraction procedure was retained but possible variations in the results were considered.

Although the validation of each method showed good results, the quantification of yohimbine in authentic bark of yohimbe and aphrodisiac products gave different responses depending on the technique employed. The LC-UV method revealed that the authentic bark of yohimbe contained 1.303 ±0.014% (m/m) of yohimbine, while 5.42 ±0.15% (m/m) and 11.51 ±0.21% (m/m) were obtained with the LC-APCI-MS and LC-ESI-MS techniques, respectively. The recoveries of the spike of yohimbe samples with 70, 100 and 130% of the quantity of yohimbine determined by UV detection showed the same percent increments for both API and UV methods.

Calculation of consistent recovery (Table 0-2) demonstrated that the high quantity of yohimbine measured in the authentic yohimbe bark by the API technique was due to another substance possessing the same molecular mass and retention time. The LC-APCI-MS^n analysis of the yohimbine peak showed an $[M+H]^+$ ion at *m/z* 355.4 and a characteristic fragmentation that excluded the presence of any other substances except isomers. In order to confirm similar ways of fragmentation of yohimbine and its isomers, a FIA-APCI-MS^n analysis of yohimbine, α-yohimbine and corynanthine was performed (Table 0-3). The three epimers showed similar MS^2 fragmentation patterns and equivalent relative abundances of main fragments. On the other hand, fragmentation of the three main MS^2 protonated molecules (*m/z* 224.4, 212.4 and 114.4) exhibited equal fragment peaks but with different relative abundances. This phenomenon is well-known and numerous investigations concerning stereochemical effects displayed on mass spectra have been reported (Mandelbaum, 1983; Splitter and Turecek, 1994).

Table 0-3

m/z values (relative abundances in parentheses) of $[M+H]^+$ ions and characteristic fragments from LC-APCI-MS^3 analysis of yohimbine, α-yohimbine and corynanthine. The collision energy for every event was set to 30%.

	MS^1	MS^2 [355.4]			MS^3 [355.4 → 224.4]					MS^3 [355.4 → 212.2]				MS^3 [355.4 → 144.3]
Mass losses		*-131*	*-143*	*-211*	*-18*	*-32*	*-44*	*-50*	*-78*	*-18*	*-32*	*-50*	*-78*	*-27*
yohimbine	355.4	224.4 (20)	212.4 (100)	144.4 (30)	206.2 (40)	192.4 (100)	180.4 (10)	174.4 (20)	146.4 (20)	194.4 (100)	180.4 (80)	162.4 (45)	134.4 (40)	117.4 (100)
α-yohimbine	355.4	224.4 (20)	212.4 (100)	144.4 (30)	206.2 (10)	192.4 (100)	180.4 (10)	174.4 (10)		194.4 (100)	180.4 (50)	162.4 (10)	134.4 (10)	117.4 (100)
corynanthine	355.4	224.4 (20)	212.4 (100)	144.4 (20)	206.2 (60)	192.4 (100)	180.4 (60)	174.4 (20)	146.4 (70)	194.4 (20)	180.4 (100)	162.4 (45)	134.4 (20)	117.4 (100)

In order to investigate an eventual stereochemical discrimination during ionization, the same amount (500 ng on column) of standards (yohimbine, α-yohimbine and corynanthine) was injected under identical LC-UV-APCI-MS and LC-UV-ESI-MS conditions. The LC-UV chromatograms showed peaks of identical UV profile and absorbance for the three isomers but α-yohimbine had a retention time of 17.86 min. Thus, α-yohimbine did not interfere with the other two epimers that had the same retention time (13.63 min). APCI- and ESI-SIM traces (*m/z* 355.4) of yohimbine and corynanthine revealed a significant difference between their peak intensities. With the same quantity of injected standard, corynanthine showed with both APCI-MS and ESI-MS ionization intensities 3.8 and 4.2 times higher than those of yohimbine, respectively. This phenomenon probably took place during the ionization and could be the result of a stereochemical discrimination between the two isomers in the volatile phase. Epimeric differentiation was noted from the change of the relative abundances of the protonated molecules as well as from fragment ions. The difference of ionization intensities obtained by the two API techniques was possibly due to the softer ionization mode of ESI with respect to APCI. As a consequence of this observation, the presence of minimal quantities of other yohimbine isomers with similar characteristics to corynanthine could also influence the analysis. As reported earlier, yohimbe bark contains different amounts of yohimbine isomers depending on the analyzed batch. Examined aphrodisiac products showed 0.5 to 30% of variations in the calculated yohimbine amount between the UV and API-MS methods. This dissimilarity demonstrated the impossibility of employing LC-API-MS methods for a multibotanical preparation containing authentic yohimbe bark or its extracts. On the other hand, the UV and API-MS responses were found to be very consistent for multibotanical preparations containing only yohimbine and no other epimers, for example after spiking of herbal preparation with pure yohimbine. As consequence of all these observations, it was decided to use the LC-UV-APCI-MS^n method for the quantification of yohimbine, due to the non-discriminative UV detection in conjunction with the high selectivity of the APCI-MS^n technique. Even if APCI-MS^n could make a stereochemical discrimination in yohimbine epimer ionization, the fragmentation patterns of the different isomers would be the same making it possible to detect compounds contaminating the yohimbine peak. The use of this method was not affected by the presence of epimers with same retention time as yohimbine, because of their relatively small quantities. Moreover, their residual presence has not a significant pharmacological influence as they have similar effects. Corynanthine also is an α_2-adrenoceptor inhibitor but much less potent than yohimbine (Howe and Yaksh, 1986).

Results of the analysis of aphrodisiac products are presented in Table 0-4. Products 1-5, 19 and 20 were not commercialized as aphrodisiacs containing yohimbine and absence of trace amounts of this compound confirmed the declaration on product labels. Products 6 and 7 were aphrodisiacs bought from Zimbabwe supposedly containing yohimbe bark. The fact that *P.*

yohimbe is a plant native to West Africa and not to the East of this continent, was source of doubt about the presence of this plant in the aphrodisiac preparations. The qualitative analysis showed total absence of yohimbine in these two products, corroborating the suspicion previously articulated. Products 8 to 18 were commercialized as aphrodisiacs containing yohimbe bark or yohimbe extract, but in general, as illustrated in Table 0-4, the product labels lacked precision about their yohimbine content. Some labels declared that the product contained yohimbe without specifying if it was the authentic bark or an extract of it. Other labels did not indicate the type of extracts (aqueous, organic, alkaline, etc.) and their yohimbine percentage. Product 10 did not declare yohimbe on the label, and even worse, product 18 was sent from the producer without a label on the bottle, justifying this by informing that customs did not allow 2 ingredients into the country. Only two products (9 and 11) declared explicitly the amount of yohimbine (4 mg and 2.7 mg per tablet, respectively) but this did not correspond to the quantity experimentally found (5.8 mg ± 1.1 and 1.24 mg ± 0.14, in that order).

Seven products examined in this study showed relatively low yohimbine levels, going from 0.504 mg ± 0.080 to 1.24 mg ± 0.14 per unit. Such quantities would probably cause a modest health risk; in fact the maximal dose prescribed by directions on labels went from 1.18 mg ± 0.19 to 3.52 mg ± 0.66 of yohimbine per day. Even though the potential health risks caused by these aphrodisiacs containing yohimbine are low, this does not mean that the use of these products is completely safe. In effect, these commercial products were analyzed only for their yohimbine content and not for other dangerous compounds. Anyway, the presence of low quantities of active principle in these herbal medications did not lead to the therapeutic effect sought. This could be the cause of unnecessary frustrations and economic costs for the patient. Despite these results, four analyzed aphrodisiac products (9, 12, 14 and 18) showed a yohimbine amount that could pose severe health risks. Although the quantity of yohimbine in product 9 was declared as earlier described, the relatively high maximal dose per day prescribed on the label (23.2 mg ± 4.5) could be dangerous especially for patients with blood pressure problems or taking other medications. The yohimbine amount in product 12 was also high and in addition the suggested use did not limit the intake (19.0 mg ± 1.0) of a maximal dose per day. Product 14 had a maximal dose per day prescribed (11.64 mg ± 0.31) which was lower than the last cited products but not declaring the quantity of yohimbine could create dosage problems, even if used as directed on label. The use of product 18 could result in a serious health risk because of its yohimbine amount (3.15 mg ± 0.34 per tablet) and above all for not having a label with ingredients and directions on the bottle. Moreover, the presence in this mixture of two ingredients not allowed by the Swiss drug registration authorities drastically increases the health risks for the patient.

Table 0-4

Aphrodisiac products analyzed showing corresponding dosage forms, directions on labels, amount of yohimbe bark, yohimbe extract or yohimbine declared, and content of yohimbine as measured using the described methods.

	Product	Dosage form	Directions	Yohimbe or yohimbine per unit declared on label	Yohimbine per unit determined [mg]	Yohimbine per day [a] [mg]
1	Vuka vuka Herbal Tea	Powder	2-3 teaspoons / day	None	0	0
2	Vuka vuka	Capsules	1-3 x 3 / day	None	0	0
3	Vuka vuka	Tablets	1-2 x 2 / day	None	0	0
4	Vuka vuka	Liquid	N/A	None	0	0
5	Extra-Plus (Vuka vuka)	Capsules	1-2 / day	None	0	0
6	Vuka Nkuzi	Capsules	1 x 2 / day	N/A	0	0
7	Vuka vuka	Powder	N/A	N/A	0	0
8	Erectol	Capsules	2 x 1-2 / day	150 mg yohimbe	0.504 ± 0.080	2.02 ± 0.32
9	Milagro	Tablets	2 x 2 / day	200 mg standard yohimbe extract (2% yohimbine)	5.8 ± 1.1	23.2 ± 4.5
10	Stamina Chi	Liquid	1 mL x 3 / day	N/A	0.99 ± 0.18	2.97 ± 0.55
11	Virile 1	Tablets	2 / day	2.7 mg yohimbine (from 270 mg standard yohimbe extract)	1.24 ± 0.14	2.47 ± 0.27
12	Virile-max (Male formula)	Capsules	2 at 1-2 h BI [b]	250 mg yohimbe extract (2% yohimbine)	9.52 ± 0.52	19.0 ± 1.0
13	Super Herbal V	Capsules	1 x 2 / day	125 mg yohimbe bark	0.59 ± 0.10	1.18 ± 0.19
14	Male-Repro	Solid	1-5 teaspoons / day	yohimbe	2.329 ± 0.062	11.64 ± 0.31
15	Endow Plus (for men)	Capsules	4 at 45 min-2 h BI	yohimbe	0.88 ± 0.16	3.52 ± 0.66
16	Herbal V	Tablets	2 at 1h BI	125 mg yohimbe extract (2% yohimbine)	1.21 ± 0.18	2.43 ± 0.36
17	Virility-V (Herbs for Life)	Tablets	2-3 / day or at 1h BI	250 mg yohimbe extract (2% yohimbine)	0.5262 ± 0.0045	1.579 ± 0.013
18	UVP - Male formula	Tablets	1-4 at 30 min BI	N/A	3.15 ± 0.34	12.6 ± 1.4
19	Bois bandé des caraïbes	Liquid	20 drops BI	None	0	0
20	Kama Sutra	Tablets	3 at 1h BI	None	0	0

[a] Calculated from the dose prescribed in the directions for use (single daily use assumed where appropriate) using the yohimbine content determined.
[b] BI = before intercourse.

IV. Phytochemical investigation of *Erythroxylum vacciniifolium* used in Brazilian traditional medicine as an aphrodisiac

1. Introduction

1.1. Catuaba: a popular Brazilian aphrodisiac

For over a century, *catuaba* has been one of the most famous herbal medicines in Brazil. In recent times, it has been the focus of great worldwide public interest because of the use of the bark as an aphrodisiac and a remedy for erectile dysfunction. In the mid-nineteenth century, *catuaba* was a popular remedy, and from the early part of the last century it has been sold in Brazilian herbal markets. Nowadays, different *catuaba* "wines" are sold on supermarket shelves in many regions of the country and many websites advertise miraculous natural aphrodisiacs containing the bark of this plant. People of Northeastern Brazil (Piauí, Bahia, Pernambuco, and Paraíba) still utilize *catuaba* as an aphrodisiac or tonic, and for other purposes (Daly, 1990; De Almeida, 1993). Moreover, it has been attributed in traditional medicine with curing some rather unusual ailments, including neurasthenia, hypochondria, and nervous insomnia (De Almeida, 1993; Teixeira da Fonseca, 1922).

The meaning of *catuaba* was first referenced in 1860 by Freire Alemão who indicated that this name in the Tupi language was a compound word meaning "homem válido" ("true man") (Pereira, 1978). Other interpretations support that the name *catuaba* probably comes from língua geral, a predominantly Tupi-Guarani language developed by the Jesuits, meaning "good leaf" or "good tree" ("árvore boa") (Pío Corrêa, 1931). This name is a polymorphic descriptive term with two components, the first of which, *Katu*, indubitably means "good". The second component of the word could be interpreted as "leaf" (*-rába*), "tree" (*-íba*), or "person" (*-abá*). Even if neither of these interpretations corresponds perfectly, it is most likely that "good leaf" is the true meaning for *catuaba* (Daly, 1990). Another name given to this plant by Pío Carrêa is *catuíba* corroborating the "good tree" interpretation.

Despite its popularity, no one seems to be able to identify with certitude the plant corresponding to *catuaba*. Depending on the source, plants of different genera, from the Erythroxylaceae, Bignoniaceae, Sapotaceae, Euphorbiaceae, Myrtaceae, Meliaceae, Apocynaceae, or Burseraceae, have been referred to as *catuaba*. The identification problems have been complicated by two factors. First, the plant is known as an aphrodisiac and, as other "magic potions", has a certain air of mystery around it, and, as a consequence, the taxonomic ambiguity. Second, the movement over the century of Northeastern Brazilians due to several catastrophic droughts has resulted in the spread of Northeastern culture and traditional medicine through other parts of the country. Originally, the name *catuaba* corresponded to a single species, but the displacement of Northeasterners extended the folk term to other species. As a

result, the name *catuaba* corresponds to different species in different regions, even if the uses are the same.

The identification of the original plant corresponding to *catuaba* has called the attention of many Brazilian botanists over the years. One of the greatest, Adolpho Ducke, dedicated much time to this identification problem as reported in his last paper published posthumously (Ducke, 1966). The confusion about *catuaba* identity has been maintained and even increased by inaccurate publications on economic plants. Sometimes, these publications fail to cite evidence or references, and report identifications without citing vouchers. Once mistakes are in the literature, it is very difficult to correct them and they can become the origin of other misinterpretations. As an example, in the Northeastern region of the country, *catuaba* has been referred to different *Erythroxylum* species and some authors erroneously stated that all these species corresponded to *E. catuaba*. This *nomen nudum*[§] has been inaccurately reported by different authors over the years and its employment still generates some confusion. Additional misunderstandings have been created from a doctoral thesis written by A. J. Silva in Salvador in 1904 (Silva, 1904). According to Ducke (1966), this PhD student was the first to report the identification of *E. catuaba* but unfortunately the description of the plant is a composite of three distinct species. Ducke concluded that the described plant in the Silva's thesis did not even belong to the *Erythroxylum* genus but was probably a *Trichilia*.

Ducke spent the last years of his life in the Northeastern Brazil writing about the flora of this region. Among other plants, he identified *Erythroxylum* specimens collected in the Ceará region and in particular, in Serra do Araripe. A plant locally known as *catuaba* was recognized by the famous botanist as *E. vacciniifolium* Mart. Another botanist, Monteiro Silva, also identified in this area a plant called *catuaba* as an *Erythroxylum*, even though he used the *nomen nudum E. catuaba* (Silva, 1951). He also reported detailed instructions for the preparation of tinctures and decoctions of this plant by traditional healers. In the early 1980s, during one of his research field trips in Serra do Araripe, the great *Erythroxylaceae* specialist T. C. Plowman noted that the bark of adult individuals of *E. vacciniifolium* Mart. had been stripped. Later, it was discovered that this bark was used by local populations as part of their traditional health care systems and particularly as an aphrodisiac. In an ethnobotanical study of an area in the interior of the Northeastern state of Piauí, L. Emperaire found that a plant locally called *catuaba preta* ("black catuaba") was an *Erythroxylum* and it was used as an aphrodisiac and a hallucinogen (Emperaire, 1983). In 1983, during an ethnobotanical study in Goiás state, M. J. Balick discovered that the Apinajé Indians were using a plant named *catuaba* as an aphrodisiac. The Indians used the bark of this plant and they drank a decoction or an alcohol infusion. Vouchers of this plant were subsequently identified by T. Plowman as *E. subracemosum* Turcz.

[§] Latin term (plural *nomina nuda*) referring to a name that has been published or mentioned without a proper and complete description or lacks a type species.

By accessing most of the evidence presented above, it is possible to conclude that the folk name *catuaba* corresponds to different species depending on the region. In Northeastern Brazil, *catuaba* is mainly referred to as a plant of the *Erythroxylum* genus and two principal species are recognized with this folk term: *E. vacciniifolium* Mart. and *E. subracemosum* Turcz. (Daly, 1990).

1.2. Characteristics of Erythroxylaceae

1.2.1. Botanical aspects

Erythroxylaceae can be recognized by their leaves, stipules that are at least in part interpetiolar and usually fasciculate inflorescence with rather small flowers; both calyx and filaments persist at the base of the fruit.

Family classification. Recent classifications have placed the Erythroxylaceae in the Linales, which includes the Hugoniaceae , Humiriaceae, Ixonanthaceae, and Linaceae (Cronquist, 1988; Takhtajan, 1997; Thorne, 1992). This family is distinguished from the remainder of the order by the petals having a ligular appendage toward the base adaxially, usually only one locule of the ovary ovuliferous, disc and nectary glands absent, and the presence of alkaloids.

Table 0-1

Taxonomy of Erythroxylaceae according to Cronquist (1988).

Kingdom:	Plantae (Plants)
Subkingdom:	Tracheobionta (Vascular plants)
Superdivision:	Spermatophyta (Seed plants)
Division:	Magnoliophyta (Flowering plants)
Class:	Magnoliopsida (Dicotyledons)
Subclass:	Rosidae
Order:	Linales
Family:	Erythroxylaceae

Current efforts at a reorganization of angiosperm phylogeny, incorporating and emphasizing molecular data, have placed the Erythroxylaceae in an expanded Malpighiales order that joins elements of the Linales with the Euphorbiaceae, Flacourtiaceae, Malpighiaceae,

Chrysobalanaceae, Pandaceae, Passifloraceae, Salicaceae, Turneraceae, Violaceae, and others (Daly, in press 2003).

According to the Angiosperm Phylogeny Group classification (APG, 1998), the systematic position of the Erythroxylaceae is reported in Figure 0-1.

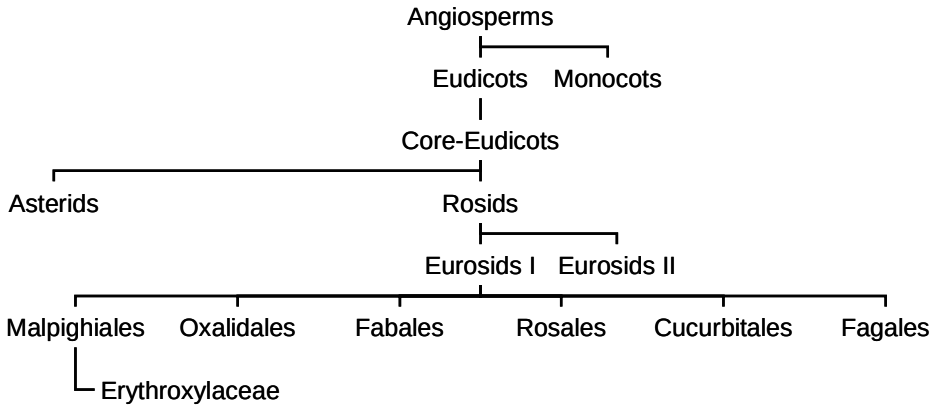


Figure 0-1

Classification of the Erythroxylaceae according to the Angiosperm Phylogeny Group (APG, 1998).

Number of genera and species. The Erythroxylaceae comprise four genera with about 250 species; three of the genera (*Aneulophus*, *Nectaropetalum*, and *Pinacopodium*), with few species (10-20), are restricted to tropical Africa. *Erythroxylum* is by the far the largest genus and occurs in the tropical part of Australia, Asia, Africa, and America. It is represented by 230 species of which about 180 are found in the Neotropics. The genus *Erythroxylum* was divided by Schulz into 19 sections (Schulz, 1907). In spite of subsequent taxonomic revisions, often engendered by the production of regional floras, Evans used these sections as a scheme for the purpose of comparative phytochemical considerations (Evans, 1981).

Distribution and habitat. Although pantropical (Figure 0-2), the Erythroxylaceae are very unequally distributed. The majority of species are in tropical America, while very few are found in the four tropical continents. *Aneulophus*, *Nectaropetalum*, and *Pinacopodium* are limited to Africa, while *Erythroxylum* occurs mostly in the Neotropics. The centers of diversity for the family are found in Venezuela, eastern Brazil and Madagascar. At least 40 species of

Erythroxylum have been recorded in Venezuela and in the Guyana region, while in Northeastern Brazil the state of Bahia alone contains at least 45 species. This genus occurs also in Ecuador, where at least 14 species, including two cultivated or naturalized, were recorded (Plowman, 1989).

Erythroxylum rarely attains high densities where the genus occurs, but it is ecologically versatile, occurring as an understory tree in low elevation rain forests, a thick-barked shrub or tree in savanna woodlands, a microphyllous shrub in arid thorn-scrub, and a membranous-leaved shrub in montane habitats (Daly, in press 2003).

Figure 0-2
Distribution map of Erythroxylaceae.

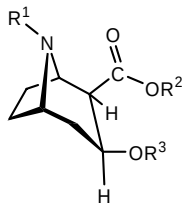
Features of the family. Glabrous shrubs or small trees, evergreen or deciduous, wood hard; sometimes spiny at the hardened tips of short shoots (*e.g.*, *Erythroxylum spinescens* Rich.); cataphylls often present, imbricate and persistent. Leaves alternate (opposite in the *Aneulophus*), usually distichous, simple; lamina margins entire; venation pinnate; vernation involute sometimes imprinting 2 parallel lines and/or a distinct central panel on the lower surface. Foliar stipules persistent on twigs or caduceus. Inflorescences axillary, sometimes short-pedunculate, composed of few-many flowered fascicles, or flowers solitary; subtended by small scarious bracteoles. Flowers actinomorphic, bisexual or seldom unisexual (plants dioecious), 5-merous, usually distylous; calyx persistent, the 5 valvate sepals united below; petals free, 5, alternate with sepals, imbricate in bud, caducous, usually appendaged on the adaxial surface; androecium with 10 stamens, in 2 whorls of 5, the outer whorl alternate with petals, united at the base by filaments and usually forming a short tube; anthers dithecal,

longitudinally dehiscent; gynoecium syncarpous, ovary superior, 3-locular or less commonly 2-locular, usually only one locule ovuliferous; placentation axile, ovules solitary, rarely 2 (in *Nectaropetalum*), pendulous, epitropous; styles 3 or less commonly 2, distinct or connate basally; stigmas capitellate, rarely sessile. Fruits small, red or purplish, drupaceous and one-seeded, rarely capsular and 2-3-seeded (in *Aneulophus*). Seeds with straight embryo, endosperm copious, rarely absent, starchy (Daly, in press 2003; Plowman, 1989; Schulz, 1907).

1.2.2. Phytochemical aspects

The phytochemical aspects of the Erythroxylaceae are represented by the phytochemical characters of the largest of four genera of the family, *Erythroxylum*. This genus, apart from the cocaine-producing species, has unexpectedly received relatively little phytochemical attention. Many constituents of different species used in traditional medicine remain unknown. Some studies, carried out over many years, indicates the occurrence, in various organs of *Erythroxylum* species, of alkaloids (principally ornithine-derived), phenylpropane derivatives, flavonoids, tannins, mono-, sesqui-, di- and triterpenoids, fatty acids and wax alcohols (Hegnauer, 1966, 1989). Among these groups, the ornithine-derived alkaloids and the diterpenoids seem to have the greatest potential as chemotaxonomic characters (Evans, 1981).

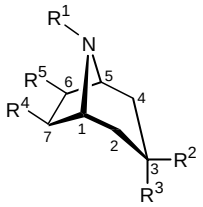
Alkaloids. The ornithine-derived moieties of *Erythroxylum* are mainly ecgonine derivatives (Figure 0-3) or hydroxytropanes (Figure 0-4) and their respective nor-derivatives. Tropine and pseudotropine alkaloids also occur in other families and are particularly widespread in the Solanaceae. Conversely, the ecgonine-type alkaloids (pseudotropine bearing an additional carboxylic group at position 2) seem to be restricted to the Erythroxylaceae. Substitution of the tropane moiety at position 2 is not known in the Solanaceae, but it does occur in the Proteaceae.



R^1	R^2	R^3	Trivial name
CH_3	H	H	Ecgonine
H	H	H	Norecgonine
CH_3	CH_3	C_6H_5CO (benzoyl)	Cocaine
CH_3	CH_3	C_6H_5–CH=CH–CO (cinnamoyl)	Cinnamoylcocaine
CH_3	H	C_6H_5CO (benzoyl)	Benzoylecgonine
CH_3	H	CH_3	Methylecgonine
CH_3	CH_3	α-truxillic acid	Cocamine (α-Truxilline)
CH_3	CH_3	β-truxillic acid	Isococamine (β-Truxilline)

Figure 0-3

Ecgonine and its derivatives occurring in the Erythroxylaceae.



R^1	R^2	R^3	R^4	R^5	Trivial name
CH_3	H	OH	H	H	3α-hydroxytropane
CH_3	OH	H	H	H	3β-hydroxytropane
CH_3	H	OH	H	OH	3α,6β-dihydroxytropane
CH_3	H	OH	OH	OH	3α,6β,7β-trihydroxytropane
H	H	OH	H	H	3α-hydroxynortropane
H	OH	H	H	H	3β-hydroxynortropane
H	H	OH	H	OH	3α,6β-dihydroxynortropane
H	H	OH	OH	OH	3α,6β,7β-trihydroxynortropane

Figure 0-4

Hydroxytropanes and respective *N*-nor-derivatives occurring in the Erythroxylaceae.

Sometimes the biosynthetically related bases hygrine, hygroline and cuscohygrine are also present (Figure 0-5), and in one instance small amounts of nicotine were observed (Hegnauer, 1981).

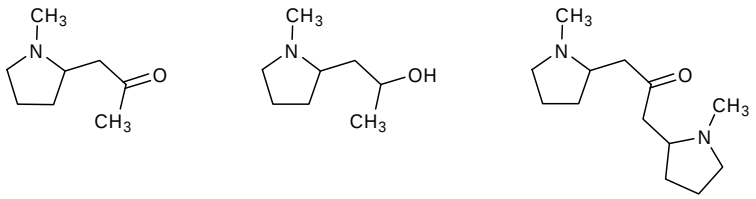


Figure 0-5

Biosynthetically related bases of tropane alkaloids in the Erythroxylaceae.

The phytochemical investigations on *Erythroxylum* species have often been restricted to the occurrence of cocaine and cocaine-derived compounds in their leaves. These alkaloids were found only in 2 species and 4 varieties: *E. coca* var. *coca* Lam. (*Bolivian* or *Huanuco coca*) and var. *ipadu* Plowman (*Amazonian coca*); *E. novogranatense* var. *novogranatense* Morris (*Columbian coca*) and var. *truxillense* Morris (*Trujillo coca*) (Plowman, 1982). In a review article, Rivier reports cocaine and the *cis* and *trans* isomers of cinnamoyl cocaine in these two species (Rivier, 1981) but the ratios between the various alkaloids served no chemotaxonomic purpose as there was great variation within the species. Among the species examined, cocaine is produced in quantity only in the cultivated varieties; wild species morphologically close to *E. coca* do not appear to contain the alkaloid (Holmstedt *et al.*, 1977). Apart from these cultivated species, traces of cocaine were found only in two plants of Venezuela, *E. recurrens* Huber and

E. steyermarkii Plowman. Cocaine does not seem to be an alkaloid of the Old World species while ecgonine derivatives have been reported for *E. dekindtii* O. E. Schulz (Alyahya *et al.*, 1979) and for *E. monogynum* Roxb. (Chopra and Ghosh, 1938).

The characteristic alkaloids of the *Erythroxylum* species are the hydroxytropanes, and the species presently investigated contains an extensive range of their esters. The tropane moieties of alkaloids within this genus, in themselves, show little use as intrageneric chemotaxonomic characters, while esterifying acids are often distinctive features of certain species (Griffin and Lin, 2000). Benzoic, cinnamic, trimethoxybenzoic, trimethoxycinnamic, truxillic (cinnamic acids dimers) and phenylacetic acids are common esterifying acids of both New and Old World species (Figure 0-6).

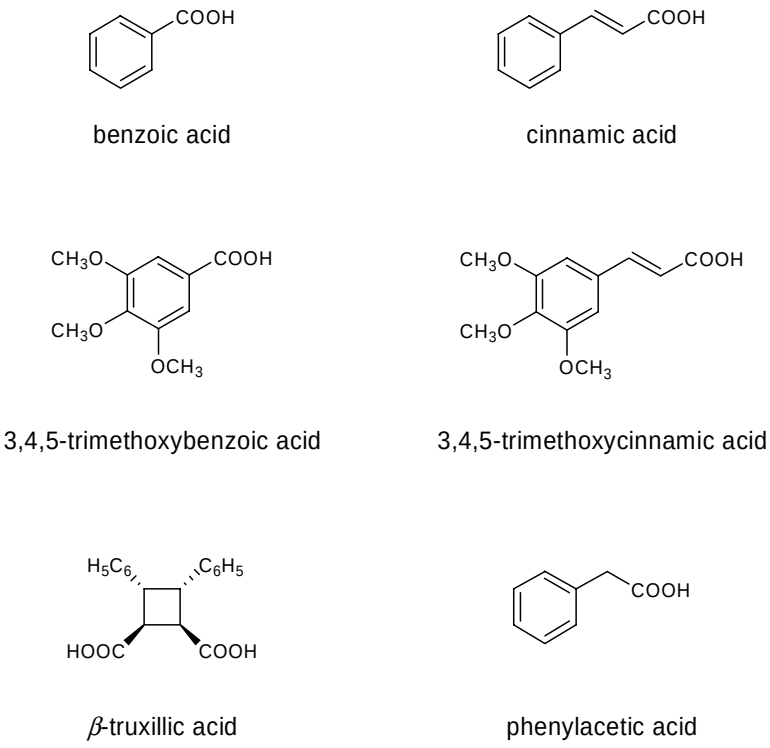
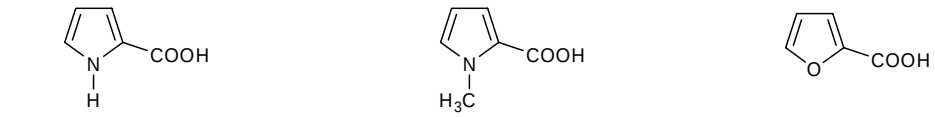


Figure 0-6

Common esterifying acids of tropane alkaloids in Erythroxylaceae.

The combination of certain of these acids for any one species is sometimes unique. In *E. australe* Muell a number of tigloyl esters are present, tiglic acid esters not yet having been isolated from any other species of the genus. Some acid moieties, such as pyrrole-2-carboxylic acids and 2-furoic acid are unique for certain species, *E. vacciniifolium* Mart. and *E. dekindtii* O. E. Schulz, respectively (Figure 0-7).



pyrrole-2-carboxylic acid *N*-methylpyrrole-2-carboxylic acid furoic acid

Figure 0-7

Unique esterifying acids of tropane alkaloids for *E. vacciniifolium* Mart. (pyrrole-2-carboxylic and *N*-methylpyrrole-2-carboxylic acids) and *E. dekindtii* O. E. Schulz (2-furoic acid).

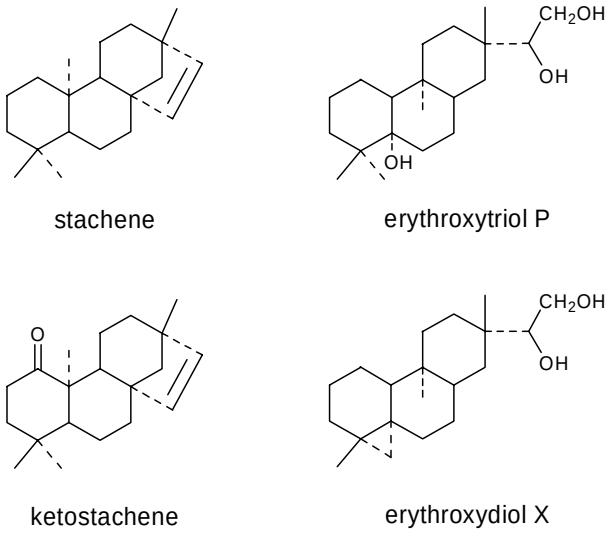
Aromatic acids. Aromatic acids are common constituents of the Erythroxylaceae. The cinnamic acids are mainly represented by caffeic acid and its derivatives such as chlorogenic acids (Hegnauer, 1981). Methyl salicylate is present in different species of *Erythroxylum* which probably contain monotropitoside- or violutoside-like glycosides. In addition, as reported before, different aromatic acids (benzoic, cinnamic, trimethoxybenzoic, trimethoxycinnamic, phenylacetic and 2-hydroxy-3-phenylpropionic acids) occur esterifying with alkamines in the alkaloid mixtures of many species of *Erythroxylum* (Evans, 1981).

Flavonols and related flavonoids. Flavonoid compounds are largely present in leaves of Erythroxylaceae and the few true phytochemical investigations suggest the prevalence of flavonols. The main flavonoid compounds are kaempferol- and quercetin-3-glycosides. Rutin, quercitrin, isoquercitrin and ombuin-3-rutinoside were isolated from different New and Old World species, and seem to be the primary flavonoids of the Erythroxylaceae. The scarcity of available facts, however, does not allow any general conclusion yet about the flavonoid chemistry of this family.

Tannins. Little is known about the true nature of tannins of Erythroxylaceae, but it seems that these phenolic compounds are more or less abundant in roots, stems and leaves. The isolation of specific types of tannins from some species suggests that Erythroxylaceae belong to those families of Angiosperms which produce condensed tannins and ellagitannins in variable amounts (Hegnauer, 1981).

Isoprenoid compounds. Whereas mixtures of essential oils and resins (*i.e.*, balsams), composed of mono-, sesqui- and diterpenoid metabolites, tend to be deposited in wood, triterpenoid compounds seem to be in leaf- and fruit-waxes of Erythroxylaceae. Unfortunately, only few species have been investigated thoroughly for a definitive conclusion.

The wood of *E. monogynum* Roxb. has proved to be a rich source of diterpenoids, including stachene and a number of its derivatives, together with a range of alcohols such as erythroxytriol P (Connolly *et al.*, 1966). Five new diterpenoids related to stachene, all of which possessed an oxygen substituent at C-1, were isolated from *E. australe* Muell (Figure 0-8) (Connolly and Harding, 1972).



stachene — erythroxytriol P

ketostachene — erythroxydiol X

Figure 0-8

Example of diterpenoids of *Erythroxylum.*

1.2.3. Economic uses

By far the most important species of the family is the coca plant, *Erythroxylum coca* Lam. Archaeological evidence indicates that coca was domesticated by 1500 BC in South America. In pre-Columbian times, coca was a major element of the economy (Hastdorf, 1987). Andean peasants and miners traditionally have consumed coca by masticating wads of leaves, combined with chalk or ash, which helps dissolve the alkaloids into saliva. Coca chewing has been used as a tonic and stimulant to allay hunger and altitude sickeness, and for other medicinal and ritual purposes. A lowland Amazonian cultivar, *E. coca* var. *ipadu* Plowman, has figured prominently in some western Amazonian indigenous cultures for similar purposes (Daly, in press 2003).

With the Spanish conquest, coca became an important commercial crop, grown in the Yungas to supply workers in the rich silver mines of Bolivia. In the late 1800's Europeans and north Americans learned about coca and begun importing it for elixirs and patent medicines (Boucher, 1991). A real commercial success of coca leaves began with the production of "Vin Mariani" which was introduced by Angelo Mariani in the 1860s and it was claimed to have stimulant,

analgesic, anesthetic, and carminative properties. Scientists like Sigmund Freud described it as a "magical drug" and encouraged family, friend, and colleagues to use it (in the book "Über Coca" by Freud). An extract of coca leaves, together with extracts of *Cola nitida* Schott et Endl. Sterculiaceae (caffeine), were primary ingredients in an even more famous beverage, Coca-Cola®. In 1904 fears about the narcotic properties of cocaine led to the deletion of coca leaf extract. Cocaine which served as the template compound for the development of numerous invaluable synthetic anesthetics including lidocaine, novocaine, and xylocaine, is still used in ophthalmic surgery. Unfortunately, the coca plant is now widely regarded as a nuisance to society, because of the distorted use on a massive scale of one of its ingredients, cocaine. In 1997, an estimated 1.5 million Americans were current cocaine users, according to the 1997 National Household Survey on Drug Abuse (NHSDA). This number has not changed significantly since 1992, although it is a dramatic decrease from the 1985 peak of 5.7 million cocaine users (3% of the population). Based upon additional data sources that take into account users underrepresented in the NHSDA, the Office of National Drug Control Policy estimates the number of chronic users at 3.6 million (NIDA, 1999).

In the Northeastern Brazil, *E. vacciniifolium* Mart. and *E. subracemosum* Turcz. are two of the plants most prominently referred to as *catuaba*, a Tupi name applied to a number of species used as tonics and aphrodisiacs (see chapter 1.1).

E. dekindtii O. E. Schulz, called Olokuto by the natives, is a small West African shrub found particularly in the mountainous ravines of Angola. A decoction prepared from the roots and leaves of this plant is used as a febrifuge (Alyahya *et al.*, 1979).

E. moonii Hochr. is a small shrub which is used in Sri Lanka as an effective anthelmintic for roundworms and for the suppuration of boils and abscesses (Jayaweera, 1980). Another plant endemic to this country is *E. zeylanicum* O. E. Schulz which is traditionally also used as an anthelmintic (Jayaweera, 1980). *E. pervillei* Baill. is an Erythroxylaceae from Madagascar. It is known locally as *Tsivano* and has several folkloric uses, including as a fish poison and to treat abdominal pain and tumors (Silva *et al.*, 2001). *E. macrocarpum* O. E. Schulz and *E. sideroxyloides* Lam. are members of the *E. laurifolium* Lam. *sensu lato* complex and are indigenous to Mauritius, Réunion and the Seychelles. In Mauritius, these small trees are known as *bois de ronde* and find use as a popular treatment for kidney disorders as well as furnishing a hard timber (Al-Said *et al.*, 1986a).

The wood of some species of *Erythroxylum* is hard, strong and durable and is valued locally for making telephone poles, supports, and furniture (Daly, in press 2003).

1.3. The tropane alkaloids

Generalities. Tropane alkaloid is a trivial name to designate alkaloids with a tropane structure. The variations in this alkaloid group are given by:

- α- or β-position of the 3-OH-substituent (tropine and pseudotropine, respectively).
- Presence or absence of carboxyl group at position C-2 (ecgonine derivatives or hydroxytropane, respectively).
- Esterification of tropane moiety with different acids (*e.g.*, benzoic , cinnamic, trimethoxybenzoic, trimethoxycinnamic, truxillic, phenylacetic acids, *etc.*).
- Additional hydroxyl groups in the tropane ring (*e.g.*, calystegines).
- Presence or absence of *N*-methyl group of tropane alkaloids (tropane or nortropane moiety).

The tropane alkaloids, such as hyoscyamine and scopolamine (Figure 0-9), are prominent and have been the focus of wide attention over the years. They were constituents of popular magical potions containing plants of the Solanaceae family, *e.g.* henbane (*Hyoscyamus niger* L.) and mandrake (*Mandragora officinarum* L.), which were used in the Middle Ages as hallucinogens. The toxic effects of thorn-apple (*Datura stramonium* L.), also known as *Jamestown weed* are well-known too. Atropine, the racemic hyoscyamine, has a potent mydriatic action and has been widely used in ophthalmics as a pupil dilatory agent. During the Renaissance fashionable ladies would drop belladonna fruit juice (*Atropa belladonna* L.) into their eyes to make themselves appear more attractive. The plant *E. coca* var. *coca* Lam. was traditionally known for its central nervous system stimulating properties and for improving endurance, and cocaine was first isolated from the leaves of this plant by Niemann in 1860. These attractive properties led to a worldwide spread of the drug which has been also used as anesthetic.

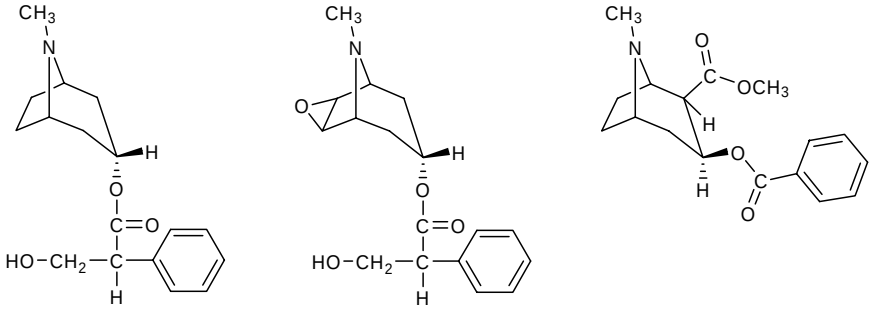


Figure 0-9

Legendary tropane alkaloids.

The structural elucidation of the tropane ring system, found in hyoscyamine and cocaine, was first secured in 1901 by Willstätter with his extensive synthetic work on this alkaloid group. The tropanes were one of the first key alkaloid, represented by coniine, nicotine, quinine, and morphine, to succumb to total synthesis. Ladenburg's synthesis of (+)-coniine was previously described in 1886 (Hesse, 2002).

The number of tropane alkaloids known from natural sources exceeds 200 (Drager, 2002). They have been isolated from different families, *e.g.* Solanaceae, Erythroxylaceae, Proteaceae, Euphorbiaceae, Rhizophoraceae, Convolvulaceae and Cruciferae, and some phylogenic trends were observed (Griffin and Lin, 2000). These families comprise mono-, di- and triesters, carboxylated and benzoylated tropanes. Recently, the location of tropanes has widened with the discovery of dimeric and trimeric forms.

Biogenesis of tropane alkaloids. Most studies in biosynthesis of tropane alkaloids have been performed on various species of *Datura* but all the available evidence suggests that similar pathways operate in other tropane alkaloid-producing plants. Because the characteristic of these alkaloids are esters of hydroxytropanes and various acids (see chapter 1.2.2) there are two distinct biogenetic moieties for each alkaloid. Generally, the amino acid ornithine, its decarboxylation products putrescine and proline constitute the basic unit of the tropine, ecgonine, nicotine (pyrrolidine ring), necine, and stachydrine groups of alkaloids.

The early discoveries in tropane alkaloid and cocaine biosynthesis must be attributed to Leete. He was the first to describe the involvement of ornithine as a precursor to the amino acid portion of the tropane ring (Leete *et al.*, 1954). Another amino acid, arginine, can clearly contribute to this pathway, as ornithine and arginine have a close metabolic relationship. As a result of feeding experiments with radiolabeled [2-^{14}C]ornithine (the label was incorporated into hyoscyamine in *D. innoxia*), Leete proposed a pathway from ornithine to putrescine through a decarboxylation (Figure 0-10). The *N*-methylation of putrescine to give *N*-methylputrescine followed by oxidative transamination would yield the 4-methylaminobutanal, a compound which is primed for spontaneous cyclisation to a N-methyl-Δ^1-pyrrolium cation. Condensation with the as yet unknown acetate-derived moiety (acetoacetic acid) would then deliver ecgonine or respectively tropinone after a decarboxylation of hygrinecarboxylic acid. Stereospecific hydrogenation of tropinone yields 3α-tropine or 3β-tropine (pseudotropine). These early experiments of Leete indicated a regiospecific incorporation of [2-^{14}C]ornithine into C-1 of the tropane moiety of hyoscyamine in *D. stramonium* L. (Leete, 1962, 1964), while later studies on this plant and other species of Solanaceae and Erythroxylaceae, have shown equal labeling into C-1 and C-5 (Hashimoto *et al.*, 1989; Leete, 1982; Liebisch *et al.*, 1963). These accumulated evidences suggest a symmetrical rather than a regiochemical incorporation.

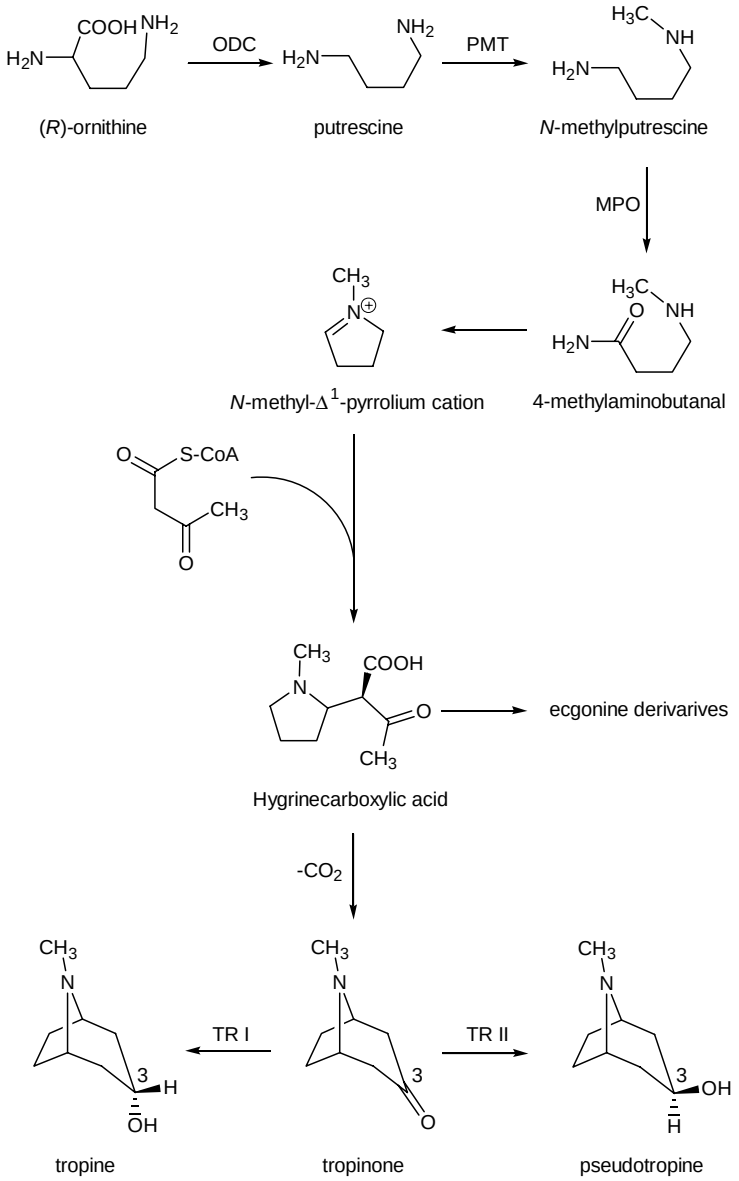


Figure 0-10

Possible biogenetic route for the tropane nucleus (Duran-Patron *et al.*, 2000). (ODC: ornithinedecarboxylase; PMT: putrescine-*N*-methyltransferase; MPO: methylputrescineoxidase; TRI: tropinonereductase I; TRII: tropinonereductase II).

Structural elucidation. The general structures of the tropane moieties are based on the following spectroscopic criteria. Esters of 3α-hydroxytropane give in their electron impact mass spectra a prominent ion, often constituting the base peak, at *m/z* 124, and correspondingly for the nor-derivative, at *m/z* 110 (Agar and Evans, 1976). Esters of the dioxygenated and trioxygenated tropane alkaloids fragment readily by loss of the C(6) –C(7) bridge to give an ion, *m/z* 113 if there is a free hydroxyl group at C-3 (*m/z* 99 for a nor-alkaloid); esterification of C-3

causes the corresponding ion to incorporate the acid moiety. By this means, the substitution pattern of the tropane ring can be established.

^{1}H-NMR spectroscopy gives significant information for the structural elucidation of tropane moieties and their esterifying acids. The structures of a di- or trisubstituted tropane core skeleton were illustrated by their typical chemical shifts (El-Iman *et al.*, 1987; Graf and Lude, 1978): a methyl linked to nitrogen (δ_H around 2.5), three or two –CH_2 (δ_H in the regions of 1.4-1.8, 2.0-2.3 and 2.7), two –CH (δ_H *ca.* 3.2-3.5) and two or three CHO– groups (δ_H *ca.* 4.0-5.8). The relative configuration of the substituent at C-3 can be established by the interpretation of ^{1}H-NMR spectrum: α-orientation gives a triplet at δ_H *ca.* 5 (*J* 5 Hz) for an ester and at δ_H *ca.* 4.0 for the free alcohol; β-orientation gives a quintet at similar frequencies with a coupling constant of *ca.* 15 Hz (Al-Said *et al.*, 1986a). The substituents at C-6 and C-7 appear to be *exo* when the corresponding *endo*-protons show coupling *J ca.* 0 Hz with the vicinal bridgehead protons (C-5 and C-1, respectively); inversely, *exo*-protons exhibit marked coupling (*J* 3-4 Hz) in similar fused systems.

1.4. Description of *Erythroxylum vacciniifolium* Mart.

Botanical aspects. Densely branched, closely spaced shrubs with rigid, sharp-tipped branches, 6 feet high, grey bark, with terete (narrowly cylindric) branches, warty, sub-distichous branchelets, the youngest flattened. Leaves short petiolate, rounded at basis and apex, mucronulate at apex, mucrone deciduous, 1-3 cm long, 0.5-2 cm broad, upper surface glaucous (blue-green) and glossy, lower surface paler, the median nerves prominent on both surfaces. Petiole 2 mm long. Foliar stipules triangular section, keeled, apex shortly aristated and bifid (two-cleft to about half way). Thin, membranous scales, the half lower part aristate, the tips deciduous, similar to stipules. Flowers produced singly, by two or three in axils of the scales. Pedicel 4 mm long, sticky on the upper part, pentangular. Sepales 2 mm long, oval-triangular, thicker at apex, lightly membraneous edge. Lanceolate, sharp and small prophylla. Doubled ligule, 4 mm long, oblong-long, wavy edge petals, with lateral anterior lobes scarcely longer than the lobe bifid on the junction, irregularly toothed margine, with the lateral posteriors lobes, three time shorter than the anterior lateral lobe, linked to a lobule, not completely squared. The staminal urceole shorter than the sepals, with a toothed pointed opening. The filaments, in the flowers with short styles, are the same length: twice longer than the staminal ureceole. In the flowers with long styles, the filaments are unequal, the shorter almost twice shorter than the urceole, and the longer almost the same size as the urceole. Ovary: ovoid, three separeted styles. Stigma: bristled, truncate at apex.

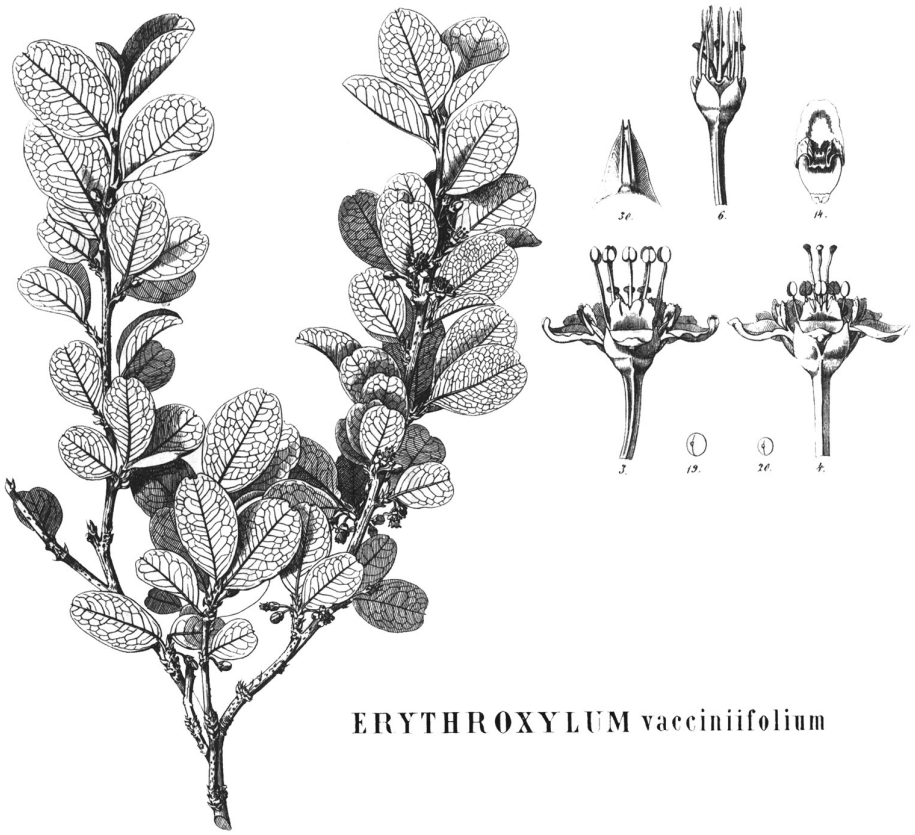


Figure 0-11

Erythroxylum vacciniifolium Mart. (Martius, 1965).

Figure 0-12

Specimen of *Erythroxylum vacciniifolium* Mart. collected in Buraquinho rain forest (João Pessoa, Paraíba, Brazil), in August 2000. The voucher specimen was deposited at the HLPQN (Herbarium do Laboratorio de Química de Produtos Naturais), Universidade Federal de Paraíba, 58059 João Pessoa, Paraíba, Brazil (JPB.-N° 152) and identified by Prof. Zoraide Maria de Medeiros Gouveia of the Department of Science of Nature, University of Paraíba, Brazil and Dr. Douglas C. Daly, The New York Botanical Garden.

Distribution and habitat. *E. vacciniifolium* Mart. is widespread in Brazil, occurring from Ceará south to Santa Catarina in a diversity of habitats (Figure 0-13). In Northeastern Brazil, this species inhabits an open vegetation type best described as “closed shrubby Caatinga”. There is little agreement on the classification of dry vegetation types in Northeastern Brazil, and different authors vary widely in their opinion. In Serra do Araripe, *E. vacciniifolium* Mart. was said to be growing in *carrasco*, a term which locally is used for closed Caatinga scrub vegetation on sandy soil.

Caatinga covers most of northeast Brazilian with an area of 300,000 square miles, interrupted by some areas of humid tropical forest. Climatic irregularity is its main characteristic, presenting a long drought period, which is reflected in the landscape. In spite of its arid and poor appearance, the Caatinga is seen as a complex ecosystem for the capacity of its various life forms to adapt to the aridity of the territory. Besides, it presents relatively fertile soils that, due to its high biodiversity, provide the area with abundant genetic resources. The formation presents heterogeneity in appearance and composition. In some places, it exhibits a short or

open forest, from which originates the name Caatinga, which means white forest in the indigenous language; in other places, the soil appears almost “nude”, showing isolated bushes. The area has two seasons: hot and dry, in the winter; and hot with rains, in the summer. During the period of drought, the vegetation has a dry aspect, without foliage, and the roots pierce the stony soil, an adaptation of the plants to the climatic conditions. In this way, the fine leaves that fall off the trees cause the plants to reduce transpiration, avoiding the loss of water, and their roots, staying on the surface of the soil, absorb the water from the rains more rapidly. In the beginning of the year, when it begins to rain, the landscape of the Caatinga changes and the gray appearance of the period of the droughts gives place to the flowers. The trees reappear full of leaves and the soil is lined with small plants.

Three other species of *Erythroxylum* have been found growing alongside *E. vacciniifolium* in the *carrasco* vegetation on the Chapada do Araripe: *E. stipulosum* Plowman, *E. rosuliferum* O. E. Schulz, and *E. loefgrenii* Diogo. These three species are restricted to dry formations in Northeastern Brazil and none of the four species occurred in adjacent cerado woodland where *E. barbatum* O. E. Schulz and *E. suberosum* A. St.-Hil. were found.

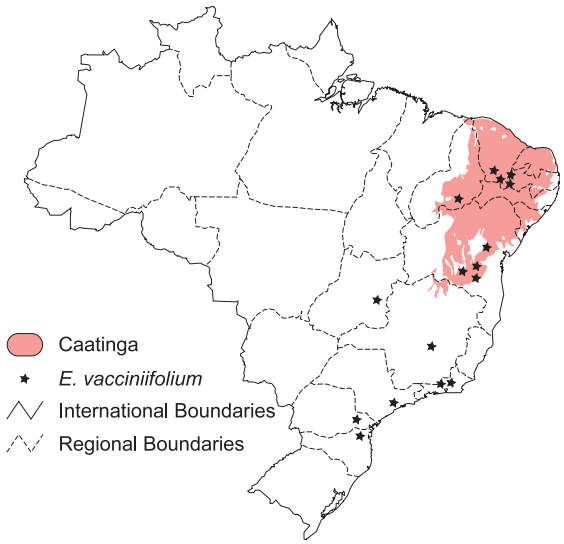


Figure 0-13

Known geographical distribution of *E. vacciniifolium* Mart. in Brazil.

Traditional uses. In Northeastern Brazil, *E. vacciniifolium* Mart. is known as *catuaba* and has been attributed in traditional medicine with aphrodisiac and tonic properties (De Almeida, 1993; Teixeira da Fonseca, 1922). The bark can be smoked, burned or drunk as a water infusion. The infusion is also used against gastrointestinal problems (Emperaire, 1983). Near human settlements, the bark is found stripped from most individuals of this species and sold in medicinal markets of Northeastern Brazil (Figure 0-14). For over a century, *catuaba* has been one of the most popular herbal remedies in Brazil, even though the use of this folk name for other species in traditional medicine has generated some confusion (see chapter 1.1).

Figure 0-14

E. vacciniifolium Mart. sold in a medicinal plant market of João Pessoa, Paraíba, Brazil (photo E. F. Queiroz, 2001).

Previous phytochemical investigations. Despite widespread commercialization of *catuaba*, only a few pharmacological and phytochemical studies have been reported on *E. vacciniifolium* Mart. and *E. catuaba* Silva. Two describe the isolation and structure elucidation of three tropane alkaloids from *E. vacciniifolium* Mart. (Graf and Lude, 1977, 1978). Another shows hot water and alkaline extracts to have an effect on human immunodeficiency virus (HIV) and suggests a medical potential of *E. catuaba* Silva against opportunistic infections in HIV patients (Manabe *et al.*, 1992).

Graf and Lude reported that the leaves and bark of *E. vacciniifolium* Mart. contain at least eight, probably eleven alkaloids (total alkaloid content 0.032%). The three main bases were isolated by percolation, extraction, column and thin-layer chromatography. These three alkaloids were previously unknown and were named catuabines A, B and C. The structures of these catuabines were determined by EI mass spectrometry, ^{1}H- and ^{13}C-NMR spectroscopy, and elemental analysis, comparing the data obtained with those of known tropane-3,6-diesters. As shown by ^{1}H-NMR spectroscopy, these alkaloids are esters of tropane-3,6-diol with an α-configuration at C-3 and a β-configuration at C-6 (Figure 0-15). 3,4,5-Trimethoxybenzoic acid (catuabines A and B) and pyrrole-2-carboxylic acid (catuabine C) are bound at C-3. *N*-Methylpyrrole-2-carboxylic acid (catuabines A and C) and benzoic acid (catuabine B) are bound at C-6. The concentration of catuabines A, B and C in the leaves of *E. vacciniifolium* Mart. was calculated by Graf and Lude as 0.01%, 0.0005% and 0.00064%, respectively.

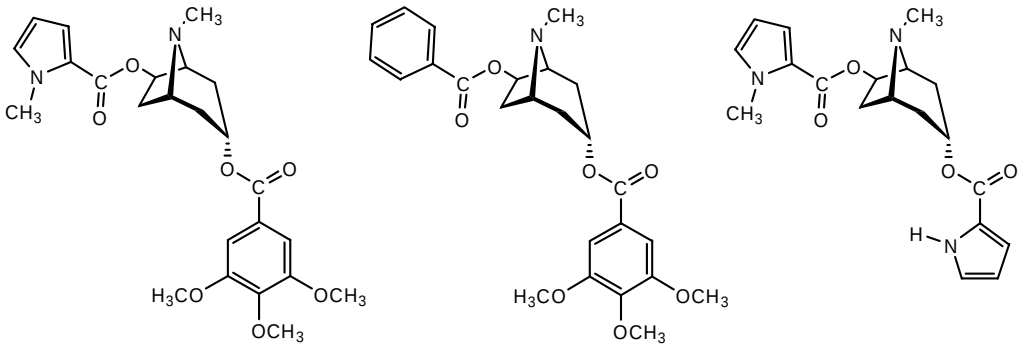


Figure 0-15

Structures of tropane alkaloids isolated from *E. vacciniifolium* Mart. (Graf and Lude, 1977, 1978).

2. Results and Discussion

2.1. Quantitative determination of total alkaloids

The quantitative estimation of total alkaloids in the bark of *E. vacciniifolium* was carried out using gravimetric and colorimetric procedures (see chapter VI.7). Gravimetric analysis is a macroscopic method involving relatively large sample amounts compared with spectrophotometric analysis. The gravimetric analysis was concerned with the weighing of total alkaloids that had been separated from other constituents by acid-base partition. Results of quantification by gravimetry are exposed in Table 0-2.

Table 0-2

Results of quantitative estimation of total alkaloids in the bark of *E. vacciniifolium* by gravimetry. An amount of stem bark powder (840.13 g) was extracted with chloroform to give 15.741 g of alkaloid extract. Three portions (a, b and c) of this homogenized extract were then used for the analysis. (b. p.: before partition; a. p.: after partition)

Sample	Weight b. p.	Weight a. p.	Alkaloid content	Average	SD	RSD	CI
a	335.6 mg	129.6 mg	0.72%				
b	330.2 mg	122.9 mg	0.70%	0.70%	± 0.03%	4.3%	± 0.07%
c	335.7 mg	120.1 mg	0.67%				

Quantitative analysis by UV spectrophotometry is based on the relationship between the degree of absorption and the concentration of the absorbing material. This relationship is valid only as long as Beer's law is obeyed. It was determined experimentally, using the same conditions that were used for subsequent analytical determinations. This was done using a calibration curve obtained by preparing several aliquots of a derived main alkaloid (catuabine D, **22**), each at different known concentrations. Consistent parameters and results of photometric quantification are reported in Table 0-3.

Table 0-3

Results of quantitative estimation of total alkaloids in the bark of *E. vacciniifolium* by colorimetry. (Abs.: absorbance; Conc.: concentration; SD: standard deviation; RSD: relative standard deviation; CI: confidence interval)

Sample	Weight	Abs.	Conc.	Alkaloid content[a]	Average	SD	RSD	CI
a	997.3 mg	0.382 UA	$2.33 \cdot 10^{-5}$ M	0.52%				
b	996.7 mg	0.387 UA	$2.38 \cdot 10^{-5}$ M	0.53%	0.53%	± 0.02%	2.9%	± 0.04%
c	982.1 mg	0.392 UA	$2.42 \cdot 10^{-5}$ M	0.55%				
			Range:	1.15-6.92·10^{-5} M				
			n:	7				
Calibration curve			Slope:	11600 ± 110				
			Y-intercept:	0.113 ± 0.0049				
			r^2:	0.9995				

[a] Alkaloid content calculated as catuabine D (**22**).

The gravimetric and colorimetric methods showed acceptable precision and gave comparable alkaloid content for the bark of *E. vacciniifolium* (0.70% ± 0.07% and 0.53% ± 0.04%, respectively). The main alkaloid catuabine D was used as standard for the calibration curve because the ratio of its molecular weight and amount in the extract was near the average of the ratio for all constituents. Even though the quantitative results were of the same order of magnitude, the two methods gave different alkaloid contents. This difference was probably a consequence of an under-estimation of total alkaloid quantification by colorimetry as the reaction between the bromocresol green indicator and the different *N*-oxide alkaloids was interfered by the supplementary oxygen atom at the N-CH_3 position of the tropane.

The tropane alkaloid content of *E. vacciniifolium* bark is relatively high compared to that found by Graf and Lude in the leaves (0.032%) (Graf and Lude, 1977). This is not surprising since the total alkaloid content of other *Erythroxylum* species or other plants of different genus containing tropane alkaloids may vary considerably depending on the analyzed organ or the period of collection. Table 0-4 illustrates some example of total alkaloid content in different *Erythroxylum* and Solanaceae.

Table 0-4

Total alkaloid content in different *Erythroxylum* and Solanaceae.

Species	Organ	Total alkaloid content
E. coca var. *coca* Lam.	Leaves	0.5-2%
E. corymbosum Boivin ex Baill.	Leaves	0.03-0.06%
E. discolor Liebm.ex O.E.Schulz	Leaves	0.35%
E. discolor Liebm.ex O.E.Schulz	Roots	0.23%
E. ecarinatum Hochr.	Leaves	0.03-0.12%
E. ecarinatum Hochr.	Stem bark	0.03%
E. gerrardii Baker	Twigs	0.1%
E. pervillei Baill.	Roots	0.2%
Atropa belladonna L.	Leaves	>0.3%
Datura stramonium L.	Leaves	>0.25%
Hyoscyamus niger L.	Leaves	>0.05%

2.2. On-line investigation of the alkaloid extract

2.2.1. Preliminary LC-UV-DAD and LC-APCI-MS^n analyses

In order to obtain a preliminary idea of the constituents present in *E. vacciniifolium*, a combined LC-UV-DAD and positive ion LC-APCI-MS analysis of the specific alkaloid extract was performed. The preparation of the alkaloid extract is reported in chapter VI.1.2. The separation was carried out using a reversed phase C_{18} column and a gradient of acetonitrile-water buffered with triethylamine (pH=11). The use of the alkaline buffer was required to increase the affinity of alkaloids to the reversed phase and to avoid charged molecules which could be responsible for hydrogen bonding and ionic interactions with silanol groups of the stationary phase, leading to additional retention and peak tailing.

In the LC-UV chromatogram (272 nm), a dozen main peaks and several minor peaks were recorded (Figure 0-16). Most of the constituents of the alkaloid extract had the same UV spectrum with a maximum of absorbance varying between 269 and 272 nm. Only three minor peaks presented different UV spectra: two of these peaks (**4** and **9**) had three maxima recorded at *ca.* 241, 271 and 329 nm; the other one (**2**) had two maxima recorded at *ca.* 241 and 294 nm. These observations suggested that almost all molecules shared the same chromophore and probably a similar core structure.

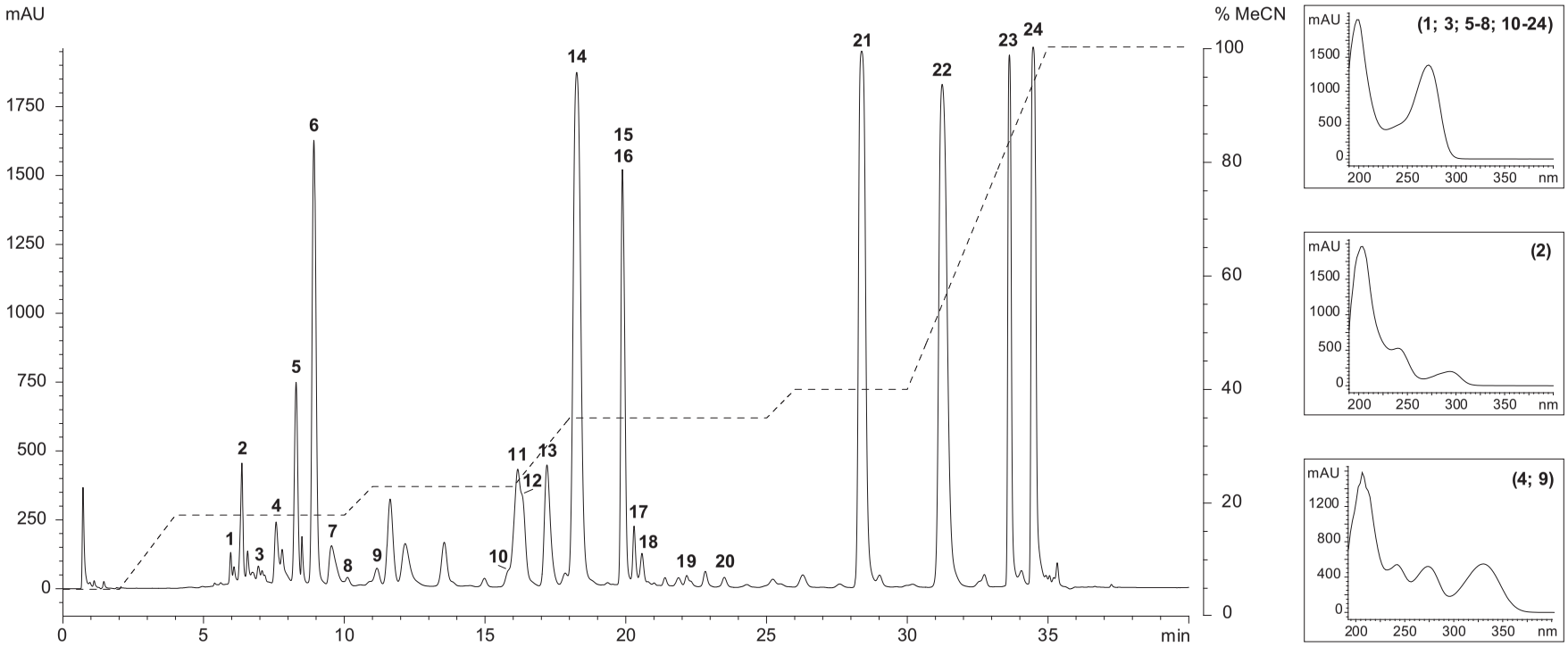


Figure 0-16

LC-DAD-UV analysis of the alkaloid extract of *E. vacciniifolium*. Chromatographic conditions: Nucleodur 100-5 C_{18} column (125 x 4.6 mm i.d., 5 μm; Macherey-Nagel) equipped with a pre-column of the same material (8 x 4.0 mm i.d., 5 μm); gradient of MeCN (+2 mM Et_3N) : water (+2 mM Et_3N) (5:95 to 100:0 in 34 min, including five isocratic elution steps each at 5:95, 18:82, 23:77, 35:65 and 40:60); injection of 50 μg; pH = 11; flow-rate 1 mL/min; detection at 272 nm. UV spectra (DAD) were recorded between 200 and 500 nm.

The LC-APCI-MS^n spectra were recorded during the same run. The total ion current trace (TIC) (Figure 0-17) was comparable to the UV trace at 272 nm. The different constituents showed protonated molecules $[M+H]^+$ in the range of 265 to 488 Da including several isomers (**1** and **3**: 297 Da; **5-8**: 281 Da; **11-15**: 265 Da; **17** and **20**: 374 Da; **18** and **21**: 388 Da) (Table 0-5). The presence of alkaloids with different numbers of nitrogen atoms was shown by even and odd *m/z* values. The MS^2 and MS^3 traces exhibited similar low molecular weight (MW) fragments for almost all detected peaks, suggesting a common main moiety for each molecule. MS^3 was needed in some cases for the recording of the characteristic low MW fragments not always observable in the MS^2 spectra.

In order to obtain additional structural information about the detected alkaloids, the observation of possible exchangeable protons (*e.g.*, N-H or O-H) was undertaken by LC-APCI-MS^n using D_2O-MeCN as eluent (Figure 0-18). In HPLC-MS analysis the exchange of ^1H_2O against ^2H_2O is commonly used for the determination of the total number of exchangeable protons in the molecule (Bringmann *et al.*, 2000b; Sandvoss *et al.*, 2000). Under these conditions all exchangeable hydrogens were replaced by deuterium. A shift of 1 Da for all constituents was due to the presence of deuterated $[M_D+D]^+$ instead of protonated molecules $[M+H]^+$. The remaining mass difference was attributable to the number of exchangeable protons of the molecules (Table 0-5). This analysis demonstrated that all detected compounds had at least one exchangeable proton, except **18**, **19**, **23** and **24**.

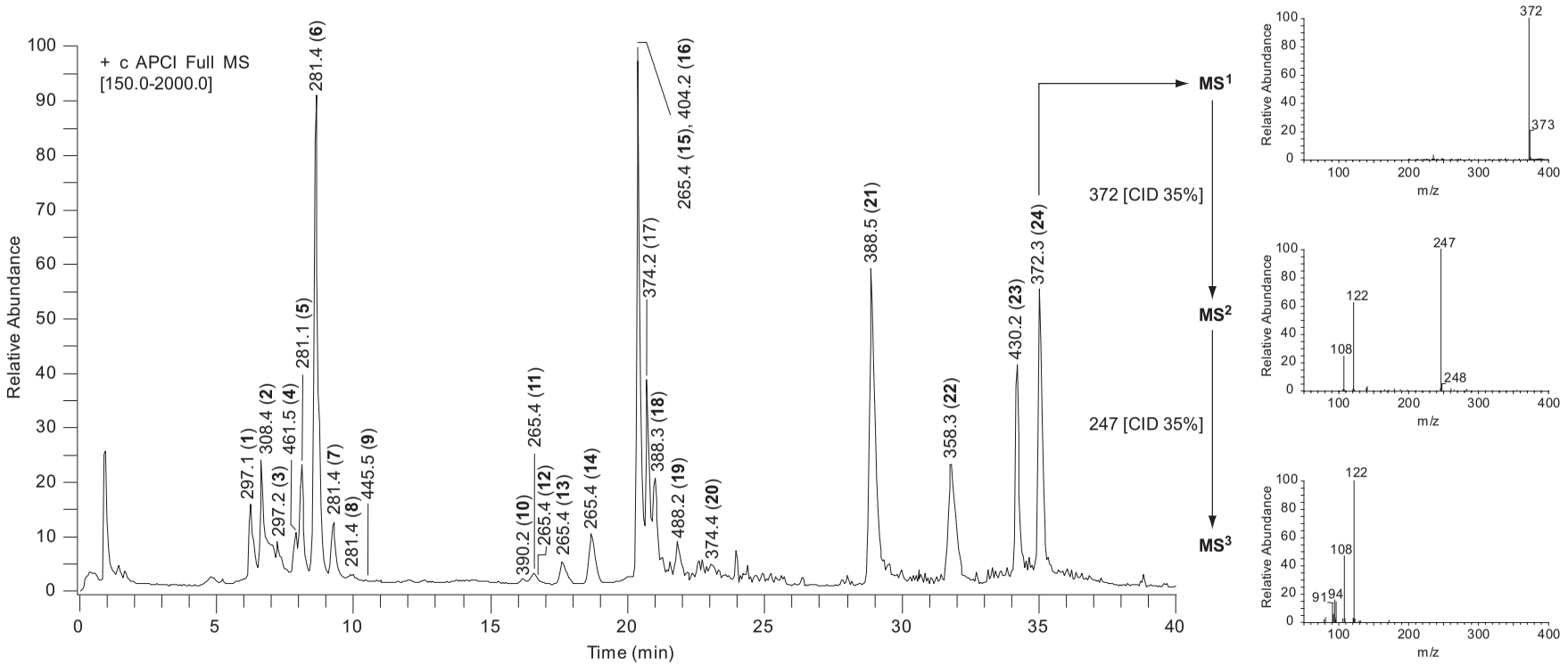


Figure 0-17

LC-APCI-MSn analysis of the alkaloid extract of *E. vacciniifolium*. MSn conditions: capillary temperature: 150°C; vaporizer temperature: 380°C; corona needle current: 6.0 μA; sheath gas (nitrogen) pressure: 60 psi; positive mode. MS^n experiments were performed by programming dependent scan events. The first event was a full MS scan (150.0-2000.0 Da) (MS^1); during the second event the main ion recorded was isolated and selectively fragmented in the ion trap (MS^2), whilst the third event (MS^3) represented the isolation and selective fragmentation of the main ion recorded in MS^2. The collision energy was set to 35%.

Table 0-5 LC-APCI-MS^n data of the alkaloid extract of *E. vacciniifolium*.

Compound	$[M_H+H]^+$	$[M_D+D]^+$	n[a]	MS^2	MS^3	MS^3
1	297	300	3	172 (100), 154 (25), 138 (85), 114 (10), 110 (15), 96 (5), 94 (15)	[297→172] 154 (85), 114 (100), 94 (30)	
2	308	312	4	156 (100), 138 (10), 110 (5), 94 (5)	[308→156] 138 (100), 120 (15), 110 (25), 96 (5), 94 (5)	
3	297	300	3	172 (100), 154 (10), 108 (5), 94 (10)	[297→172] 154 (100), 108 (5), 94 (85)	
4	461	464	3	443 (10), 263 (100), 181 (10), 138 (10)	[461→263] 245 (25), 182 (10), 138 (40), 108 (100), 96 (10)	
5	281	283	2	156 (100), 138 (20), 110 (5), 94 (5)	[281→156] 138 (95), 120 (20), 110 (20), 94 (100)	
6	281	284	3	264 (50), 156 (100), 138 (5), 112 (20), 94 (10), 82 (30)	[281→156] 138 (10), 112 (60), 94 (10), 82 (100)	
7	281	284	3	263 (30), 200 (30), 156 (60), 138 (50), 108 (100)	[281→156] 138 (100), 110 (60), 84 (50), 82 (80)	
8	281	284	3	263 (40), 156 (40), 138 (100), 108 (40), 96 (5)	[281→263] 245 (40), 138 (100), 108 (60), 96 (10)	
9	445	447	2	320 (25), 247 (100), 181 (85), 140 (10)	[445→247] 122 (100), 108 (40), 96 (20), 94 (20), 91 (20)	[445→320] 181 (100), 140 (15), 122 (40)
10	390	393	3	297 (30), 279 (50), 265 (85), 182 (75), 172 (85), 154 (25), 138 (100), 120 (10)	[390→138] 110 (100), 108 (10), 94 (15)	[390→172] 154 (100), 114 (60), 108 (50)
11	265	267	2	247 (5), 140 (100), 122 (10), 108 (10), 96 (5)	[265→140] 122 (100), 108 (20), 96 (10)	
12	265	267	2	247 (5), 140 (100), 122 (10), 108 (10), 94 (5)	[265→140] 122 (100), 108 (20), 94 (10)	
13	265	267	2	247 (5), 140 (100), 126 (35), 122 (15), 108 (5), 96 (10)	[265→140] 122 (100), 98 (30), 82 (30)	
14	265	267	2	247 (25), 203 (10), 140 (100), 122 (5), 108 (10), 96 (20)	[265→140] 122 (15), 110 (5), 96 (100)	
15	265	267	2	247 (5), 140 (100), 96 (20)	[265→140] 122 (100), 108 (5), 96 (10)	
16	404	406	2	279 (100), 182 (45), 154 (20), 138 (90), 120 (10)	[404→279] 243 (10), 154 (25), 108 (100), 94 (20)	
17	374	376	2	281 (100), 263 (20), 249 (10), 138 (5), 108 (5)	[374→281] 263 (5), 156 (100), 138 (10), 108 (5), 94 (5)	
18	388	389	1	302 (5), 263 (100), 247 (5), 202 (5), 138 (30), 122 (5)	[388→263] 245 (10), 203 (10), 138 (100), 108 (75), 94 (50)	
19	488	489	1	470 (20), 388 (100), 324 (85), 182 (10), 140 (25), 126 (30)	[488→388] 372 (100), 204 (85)	[488→324] 112 (35), 98 (100)
20	374	377	3	281 (15), 263 (100), 138 (20), 108 (15)	[374→263] 245 (15), 182 (15), 138 (30), 108 (100)	[374→281] 263 (40), 156 (70), 138 (100), 120 (50), 108 (65)
21	388	390	2	263 (100), 245 (5), 138 (15)	[388→263] 245 (10), 182 (10), 138 (35), 108 (100), 96 (10)	
22	358	360	2	265 (50), 247 (15), 233 (100), 140 (40), 122 (20), 108 (15)	[358→233] 202 (20), 140 (100), 122 (85), 96 (15), 91 (20)	[358→265] 140 (100), 122 (10), 108 (15)
23	430	431	1	388 (15), 305 (100), 245 (10), 180 (10), 138 (5), 120 (5), 108 (5)	[430→305] 263 (5), 245 (25), 180 (65), 138 (5), 108 (100)	[430→388] 263 (100), 138 (20)
24	372	373	1	247 (100), 122 (65), 108 (25)	[372→247] 122 (100), 108 (45), 96 (30), 94 (20), 91 (25)	

[a] Number of exchangeable protons (inclusive ionizing deuteron)

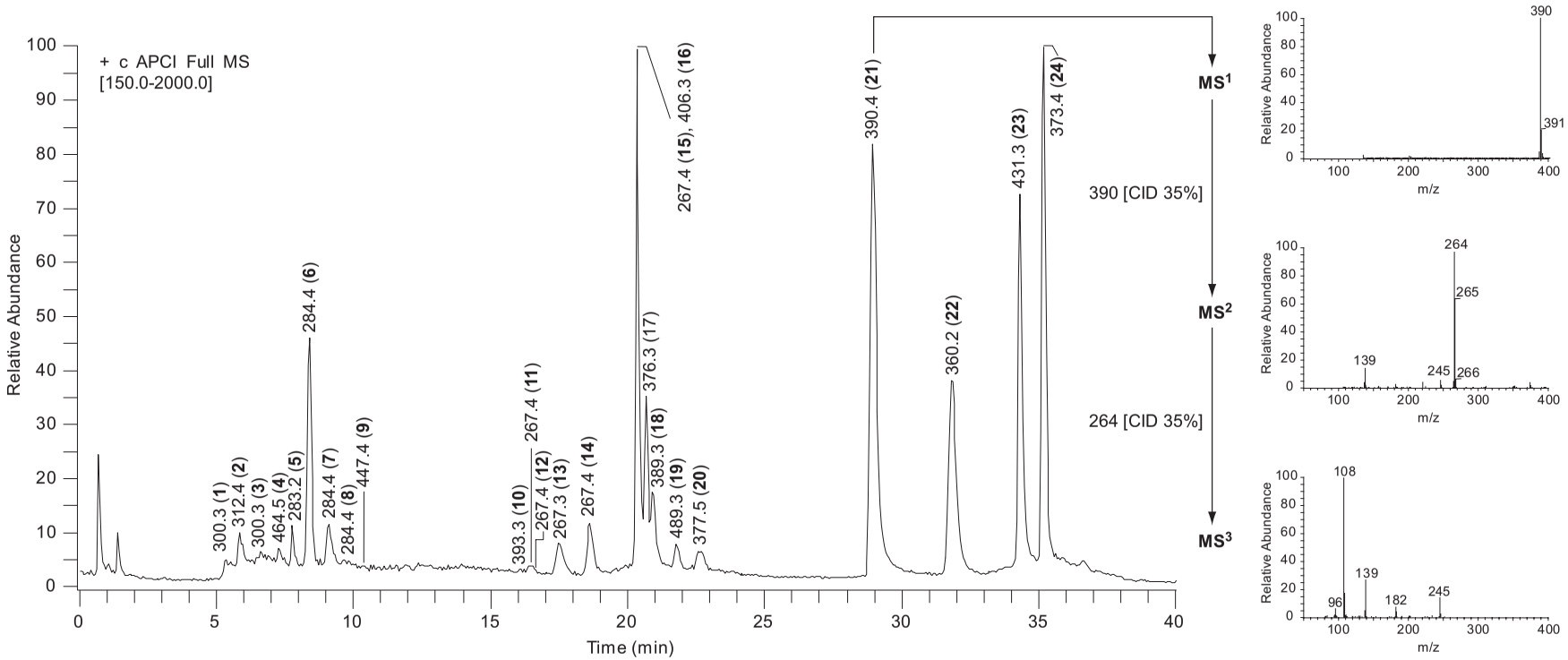


Figure 0-18

LC-APCI-MS^n analysis of the alkaloid extract of *E. vacciniifolium* using D_2O-MeCN as solvent system. HPLC and MS^n conditions as for the previous LC-UV-MS experiments.

2.2.2. High-resolution LC-APCI-TOF-MS

For an on-line molecular formula assignment, the extract was analyzed by LC-APCI-TOF-MS under identical conditions to those described for LC-MS^n. Accurate mass measurements were performed with the help of a reference compound for the lock mass. The $[M+H]^+$ of codeine at 300.1594 Da was used as a reference during the first 8 min of the HPLC analysis. Due to the instability of this ion with the increase of the MeCN concentration in the LC-gradient, the more stable $[M+H-18]^+$ codeine fragment at 282.1489 Da was then employed for the lock mass. Accurate masses could be measured for six constituents of the alkaloid extract (**1**, **2** and **21**-**24**) and for their protonated molecules $[M+H]^+$, these ranged from 297.1462 to 430.2015 Da (Table 0-6). The elemental composition of these six compounds was determined with a difference of less than 10 ppm between their measured and calculated masses.

Table 0-6

High-resolution LC-APCI-TOF-MS data of the alkaloid extract of *E. vacciniifolium*.

Compound	$[M+H]^+$ measured	$[M+H]^+$ calculated	ppm difference	Elemental composition[c]
1	297.1462	297.1445[b]	5.6	$C_{14}H_{21}N_2O_5$
2	308.1518	308.1493[b]	8.1	$C_{16}H_{22}NO_5$
21	388.1902	388.1867[a]	8.9	$C_{20}H_{26}N_3O_5$
22	358.1760	358.1762[a]	-0.5	$C_{19}H_{24}N_3O_4$
23	430.2015	430.1973[a]	9.7	$C_{22}H_{28}N_3O_6$
24	372.1954	372.1918[a]	9.6	$C_{20}H_{26}N_3O_4$

[a] Lock mass of the fragment of protonated codeine recalculated according to elemental composition $[M+H-18]^+$: *m/z* 282.1489.

b Lock mass of protonated codeine $[M+H]^+$: *m/z* 300.1594.

c Elemental composition corresponding to the $[M+H]^+$ ions; one proton has to be deduced for calculation of the molecular formula of the different constituents.

Preliminary analyses showed homogeneity in the spectral data recorded for all constituents of the alkaloid extract, which suggested close structural similarities. One of the detected compounds (**22**) was particularly interesting because of its identical molecular formula and fragmentation pattern with those reported for a tropane alkaloid esterified by two pyrrolic acids. Among the alkaloids (catuabines A, B and C) previously reported for *E. vacciniifolium* (Graf and Lude, 1978), only catuabine C matched with the on-line data recorded. The putative

detection of catuabine C in the extract and the common structural features shown by LC-UV and LC-MS, confirmed the possible presence of various related tropane alkaloids. This hypothesis was strengthened by chemotaxonomic data from the genus *Erythroxylum*, which is a well-known source of various tropane alkaloids. In order to confirm this deduction, the isolation of compound **22** was undertaken in view of its use as a reference molecule for further on-flow and stop-flow LC-NMR analyses.

2.2.3. Isolation and structure elucidation of compound 22

A single MPLC separation step performed on the alkaloid extract gave 13 fractions (A to M) (see chapter 2.3). Fraction L yielded pure compound **22** (813 mg). The structure elucidation of this compound was established by spectroscopic methods, including EIMS, HREIMS, IR, ^{1}H-, ^{13}C-, and 2D-NMR experiments (see chapter 2.4.1). Accordingly, alkaloid **22** (catuabine D) was identified as 3α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-6β-[(1*H*-pyrrol-2-yl)carbonyloxy] tropane (Figure 0-19). Thus, compound **22** was a regioisomer of catuabine C (6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-3α-[(1*H*-pyrrol-2-yl)carbonyloxy]tropane), differing by an inversion of the ester substituents.

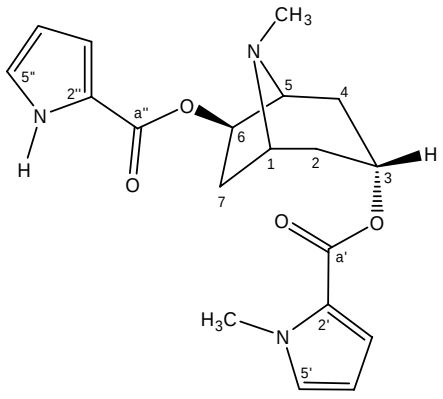


Figure 0-19

Structure of the isolated compound **22** (catuabine D).

2.2.4. On-flow and stop-flow LC-NMR

For sensitive on-flow LC-^{1}H-NMR detection, the amount of extract injected in the system was increased to 3.0 mg. The HPLC conditions were consequently optimized in order to achieve a good separation of the extract constituents. The internal diameter of the analytical column, packed with analogous C_{18} stationary phase, was increased to 8 mm. H_2O was replaced by D_2O, Et_3N by deuterated ammonia and the flow-rate was set to 1.2 mL/min for a separation over 80 min. Under these HPLC conditions, 32 scans per increment were sufficient to obtain LC-NMR spectra with good resolution. Before each set of spectra, a fast solvent suppression (WET) was run for the acquisition of LC-NMR spectra. The pulse shape for solvent suppression was calculated on fly and automatically adapted to the changes in gradient composition. With these on-flow LC-NMR conditions, the ^{1}H-NMR spectra of five main alkaloids (**14** and **21**-**24**) were successfully recorded. The proton signals of these compounds were recognizable on a contour plot of the on-flow LC-NMR experiments (Figure 0-20). This two-dimensional (2D) plot represents the usual way of processing on-flow LC-NMR data, one dimension corresponding to the NMR ppm scale and the other to the time scale. All cross peaks on this plot characterize the signal resonances of the different constituents (Wolfender *et al.*, 2001). Two other compounds (**7** and **13**), not sufficiently concentrated to be detected on-flow, were well recorded in the stop-flow mode. The interruption of the flow during the stop-flow acquisitions did not affect the LC resolution.

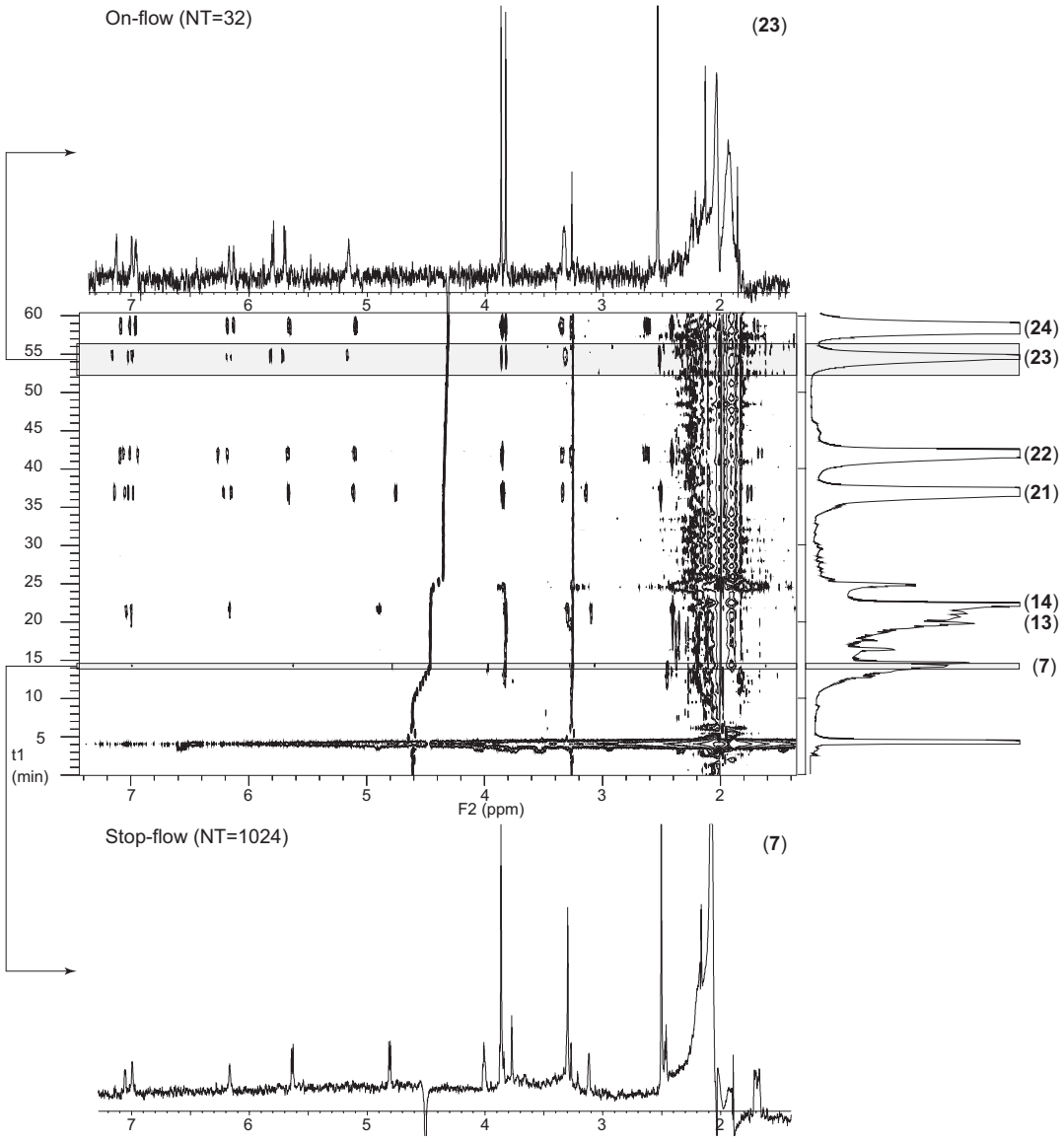


Figure 0-20

On-flow LC-NMR contour plot of the alkaloid extract of *E. vacciniifolium*. On this 2D plot all ^{1}H-NMR resonances of the analytes appear as dots. ^{1}H-NMR spectra of compounds **7** and **23** are shown as examples of resolution for on-flow and stop-flow analyses, respectively. Chromatography conditions: Nucleosil AB 100-5 C_{18} column (125 x 8.0 mm i.d., 5 μm; Macherey-Nagel) with a pre-column of the same material (8 x 8.0 mm i.d., 5 μm); gradient of MeCN (+2 mM ND_3) : D_2O (+2 mM ND_3) (5:95 to 100:0 in 80 min); injection of 3.0 mg; pH = 10; flow-rate 1.2 mL/min; detection at 272 nm. NMR conditions (Varian Unity Inova 500 MHz): ^{1}H[^{13}C] pulse field gradient indirect detection microflow probe (cell 60 μL; 3 mm i.d.); references of the solvent signal at δ 2.10 for MeCN; solvent suppression with the WET sequence; NT=32 in on-flow mode; NT=1024 in stop-flow mode.

2.2.5. Interpretation of on-line data

Correlation of ^{1}H-NMR and LC-^{1}H-NMR data of compound 22. The ^{1}H-NMR resonances of **22** obtained by on-flow LC-^{1}H-NMR differed by less than 0.2 ppm from those of the isolated compound (see chapter 2.4.1), measured in $CDCl_3$ at 30°C. This LC-^{1}H-NMR spectrum, recorded at a retention time (RT) of 42 min (Figure 0-21), showed characteristic chemical shifts associated with catuabine D (**22**). Thus, the typical resonances for a methylpyrrole substructure were exhibited by the signals at δ_H 3.96 (N-CH_3), 6.23 (H-4'), 7.05 (H-5') and 7.21 (H-3'). A non methylated pyrrole was associated with signals at δ_H 6.29 (H-4''), 7.12 (H-5'') and 7.09 (H-3''). The presence of the secondary nitrogen atom in this substructure was confirmed by the observation of an exchangeable proton by LC-MS^n analysis using D_2O as eluent (Table 0-5).

The structure of the disubstituted tropane core skeleton was illustrated by three saturated methylenes at δ_H 1.79 (H_{endo}-2), 2.30 (3H, H_{exo}-2, H_{exo}-4, H_{exo}-7) and 2.75 (H_{endo}-7). The solvent suppression around 2.10 ppm affected the signal detection of H_{endo}-4 protons. Two methine groups and one methyl group linked to the nitrogen atom were also recorded at δ_H 3.38 (H-5), 3.46 (H-1) and 2.54 (N-CH_3), respectively. The last two oxygenated methine moieties showed resonances at δ_H 5.19 (H-3) and 5.75 (H-6). The multiplicity (triplet) of the H-3 signal with the coupling constant (J = 4.4 Hz) indicated the α-orientation (*i.e.*, *endo*) of the substituent at C-3 (Agar and Evans, 1976; Al-Said *et al.*, 1989b; El-Iman *et al.*, 1987). The arrangement of the substituent at C-6 was established by the analysis of the coupling constants of H-6, H-7 and H-5 protons. The H-6 proton of the disubstituted tropane alkaloid showed two couplings (J = 7.7, 2.8) with the two H-7 protons and it did not present any coupling with the vicinal H-5 proton. This observation implied a β-orientation (*i.e.*, *exo*) of the substituent and a dihedral angle close to 90° between H-5 and H-6α (Al-Said *et al.*, 1986b; Bringmann *et al.*, 2000a).

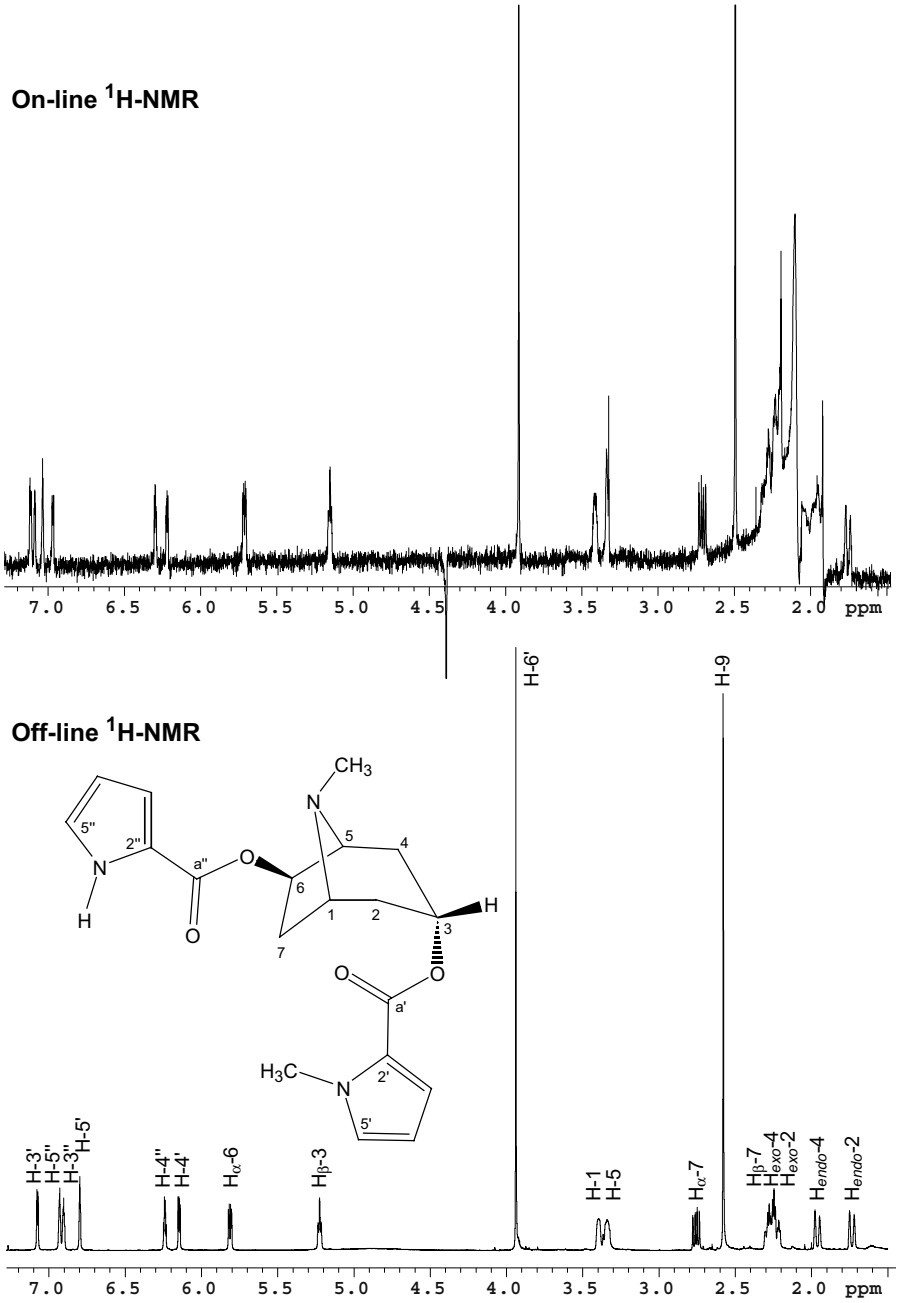


Figure 0-21

On-line and off-line ^{1}H-NMR spectra of compound **22** (catuabine D). On-line spectrum recorded at a RT of 42 min.

Structural elucidation of compounds detected by LC-^{1}H-NMR. The on-flow LC-NMR contour plot of the extract permitted a direct comparison of the chemical shifts of the other detected compounds with those mentioned for **22** (Figure 0-20). The signals exhibited great homogeneity, corroborating the hypothesis that the major constituents of the alkaloid extract of *E. vacciniifolium* were tropanic analogues of catuabine D.

The LC-^{1}H-NMR spectrum recorded for compound **24** at a retention time of 58 min exhibited similar chemical shifts to those of the spectrum of **22** (Table 0-7), supporting the presence in the molecule of a tropane moiety dioxygenated at C-3 and C-6. The difference between the two compounds was in their ester substituents. In fact, two methyl groups were recorded at δ_H 3.93 (3H, s, N-CH_3) and 3.94 (3H, s, N-CH_3) in the LC-^{1}H-NMR spectrum of **24** (Figure 0-22), indicating two methylpyrrole acid moieties. The presence of an additional methyl group in the structure of compound **24** was corroborated by the observation of its protonated molecule $[M+H]^+$, recorded at 372.1954 by LC-TOF-MS analysis, associated with a molecular formula of $C_{20}H_{25}N_3O_4$. Moreover, the LC-MS performed when using D_2O as the cosolvent confirmed the absence of exchangeable protons. On the basis of the above evidence, the structure of **24** was elucidated on-line as 3α,6β-di-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

Table 0-7

LC-^{1}H-NMR data.[a]

proton	7	13	14	21	22	23	24
1	3.18 br s	3.28 br s	3.41 m	3.45 br s	3.46 br s	3.43 br s	3.46 br s
2_{exo}	2.25 m	1.98 m	1.75 m	2.32 m	2.30 m	2.35 m	2.35 m
2_{endo}	1.75 d (15.4)	1.48 d (15.4)	1.41 m	1.88 d (16.5)	1.79 d (15.4)	1.93 d (15.3)	1.82 d (15.4)
3α	-	-	1.48 m	-	-	-	-
3β	4.08 t (4.4)	4.06 t (4.4)	1.94–2.30 m	5.20 t (4.4)	5.19 t (4.4)	5.26 t (4.4)	5.19 t (4.4)
4_{exo}	2.25 m	1.98 m	4.99 m [b]	2.32 m [b]	2.30 m [b]	2.32 m [b]	2.35 m [b]
4_{endo}	1.77 d (15.4)	1.72 d (15.4)					
5	3.34 br s	3.21 br s	3.21 d (3.3) [b]	3.25 br s	3.38 br s	3.43 br s	3.38 br s
6α	5.71 d (6.6)	5.71 dd (7.7, 2.8)		5.75 d (6.6)	5.75 dd (7.7, 2.8)	5.90 d (6.6)	5.74 dd (7.7, 2.8)
7α	4.88 d (6.6)	2.71 dd (14.8, 7.7)	1.94–2.30 m	4.85 d (6.6)	2.75 dd (14.3, 7.7)	5.80 d (6.6)	2.74 dd (14.3, 7.7)
7β	-	2.03–2.06 m	1.94–2.30 m	-	2.30 m	-	2.35 m
N-CH_3	2.57 s	2.53 s	2.53 s	2.63 s	2.54 s	2.64 s	2.54 s
	Mpc	**Mpc**	**Mpc**	**Mpc**	**Mpc**	**Mpc**	**Mpc**
3'	7.13 dd (3.9, 1.1)	7.10 dd (3.9, 2.2)	7.11 dd (3.9, 2.7)	7.21 dd (3.9, 2.2)	7.21 dd (3.9, 2.2)	7.23 dd (3.9, 1.7)	7.15 dd (3.9, 1.7)
4'	6.24 dd (3.9, 1.1)	6.23 m	6.24 dd (3.9, 2.7)	6.30 dd (3.9, 2.7)	6.23 dd (3.9, 2.7)	6.27 dd (3.9, 2.7)	6.26 dd (3.9, 2.7)
5'	7.06 dd (3.9, 1.1)	7.06 m	7.06 m	7.09 m	7.05 m	7.06 m	7.03 m
N-CH_3	3.93 s	3.93 s	3.93 s	3.95 s	3.96 s	3.92 s	3.93 s
				Mpc	**Pc**	**Mpc**	**Mpc**
3"				7.12 dd (3.9, 2.2)	7.09 dd (3.9, 2.2)	7.09 m	7.08 dd (3.9, 1.7)
4"				6.23 dd (3.9, 2.7)	6.29 dd (3.9, 2.7)	6.23 dd (3.9, 2.7)	6.21 dd (3.9, 1.7)
5"				7.05 m	7.12 dd (3.9, 1.7)	7.06 m	7.03 m
N-CH_3				3.96 s	-	3.96 s	3.94 s
						CH_3COO	
						2.24 s	

[a] δ values given in ppm, *J* values in parentheses given in Hz.
[b] the solvent suppression of acetonitrile and water respectively affected the signal detection.

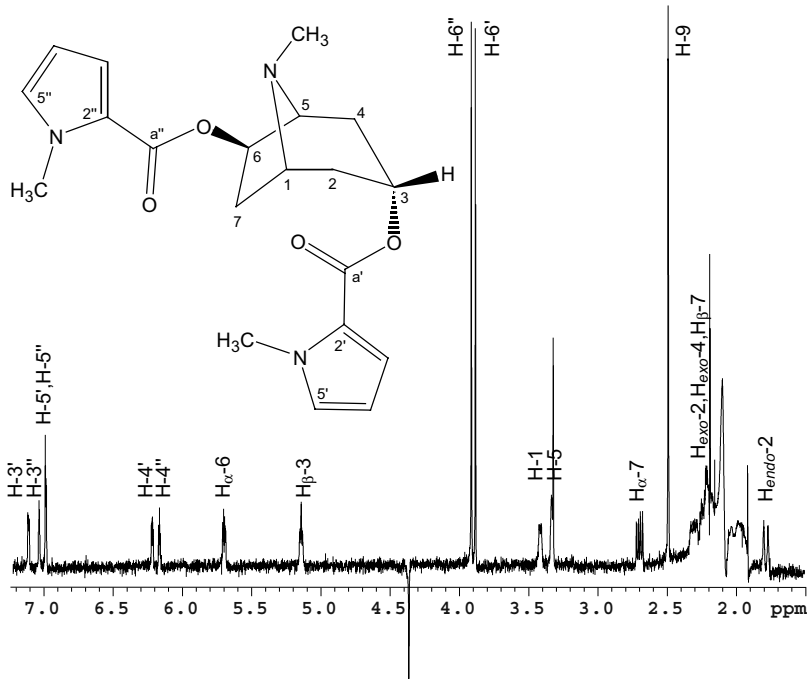


Figure 0-22

On-flow LC-^{1}H-NMR spectrum of compound **24** (RT = 58 min).

Spectral data of **21** (RT = 37 min) showed that its structure was very close to that of compound **24**, with the presence of a tropanic nucleus and two methylpyrrole ester groups (Table 0-7). However, compound **21** had an additional hydroxyl group at C-7, as indicated by the extra deshielded signal at δ_H 4.85 (H-7) and the absence of the resonance around δ_H 2.75 associated previously with the saturated methylene moiety at the 7-position for compounds **22** and **24** (Figure 0-23). The lack of any coupling constant with vicinal protons H-5 and H-1 suggested an α-orientation of protons at 6- and 7-positions, respectively. The hydroxyl group was also confirmed by the presence of one exchangeable proton in the deuterated molecule ($[M_D+D]^+$ 390 Da) when compared to the corresponding protonated molecule ($[M+H]^+$ 388 Da). The molecular formula $C_{20}H_{25}N_3O_5$ obtained by LC-TOF-MS analysis confirmed the structure elucidation of **21** as 3α,6β-di-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-7β-hydroxytropane.

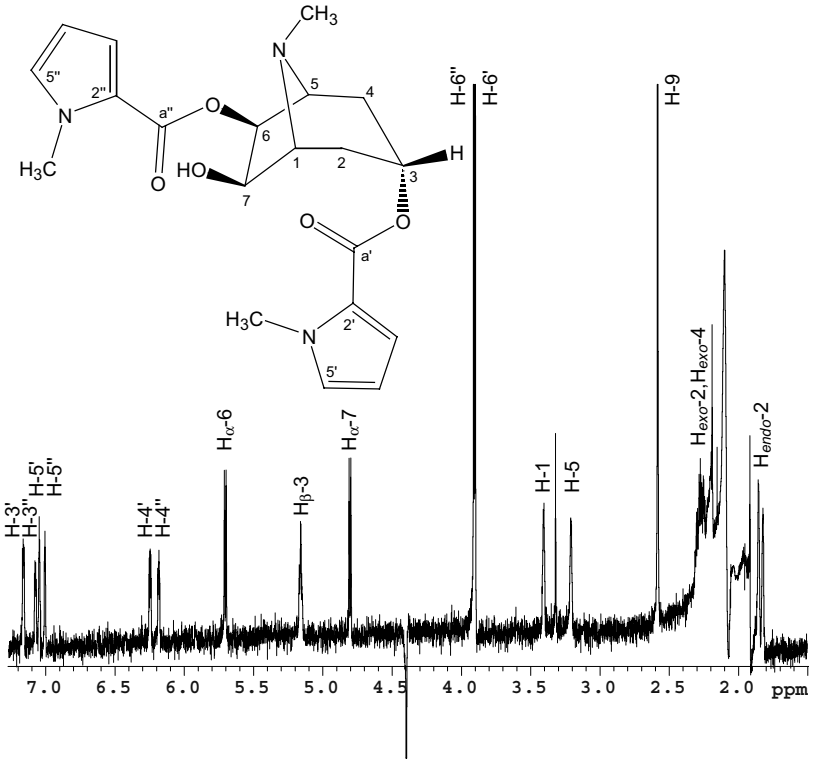


Figure 0-23

On-flow LC-^{1}H-NMR spectrum of compound **21** (RT = 37 min).

LC-TOF-MS analysis of compound **23** exhibited a protonated molecule at $[M+H]^+$ 430.2015 Da indicating a molecular formula of $C_{22}H_{28}N_3O_6$ and implying a supplementary C_2H_2O element when compared with **21**. Furthermore, the presence of a trioxygenated tropanic skeleton and two methylpyrrole ester moieties was deduced by comparison of its LC-^{1}H-NMR spectrum (RT = 54 min) with those of compound **21**. The significant differences between the two compounds were an additional resonance for one methyl group at δ_H 2.24 and a major variation ($\Delta\delta_H$ + 0.95) in the chemical shift of the signal associated with H_{endo}-7 (Figure 0-24). The downfield shifted signal suggested here the occurrence of an ester substitution in the 7-position (Al-Said *et al.*, 1986a; El-Iman *et al.*, 1987; Payo-Hill *et al.*, 2000). According to the molecular formula and the additional methyl group mentioned above, only an acetyl moiety could be located at this position. The presence of this acetyl group was also confirmed by the loss of 42 Da in the LC-MS^2 spectrum. Compound **23** was thus elucidated as 7β-acetoxy-3α-6β-di-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

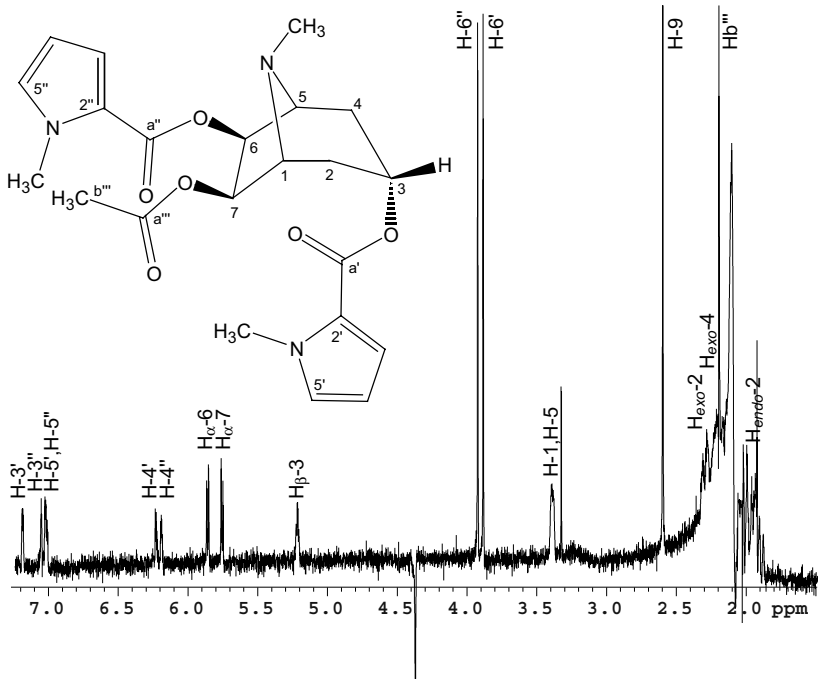


Figure 0-24

On-flow LC-^{1}H-NMR spectrum of compound **23** (RT = 54 min).

The stop-flow LC-^{1}H-NMR data (RT = 14 min) of compound **7** showed characteristic resonances for trisubstituted tropane alkaloids, as in compound **21**, but here a single methylpyrrole ester was present (Figure 0-25). The absence of the second methylpyrrole group was confirmed by the observation of a protonated molecule at 281 Da deduced from LC-MS^n experiments (Table 0-5). The similar chemical shift to other compounds associated with H_{endo}-6 at δ_H 5.71 supported the substitution of this single ester at the 6-position. The signal at δ_H 4.08, highfield shifted with respect to esterified compounds such as **21** or **23**, indicated a hydroxyl group at C-3 (Al-Said *et al.*, 1986a; El-Iman *et al.*, 1987; Payo-Hill *et al.*, 2000). The observation of two exchangeable protons confirmed the presence of two OH groups in the alkaloid. Thus, the structure of compound **7** was elucidated as 3α,7β-dihydroxy-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

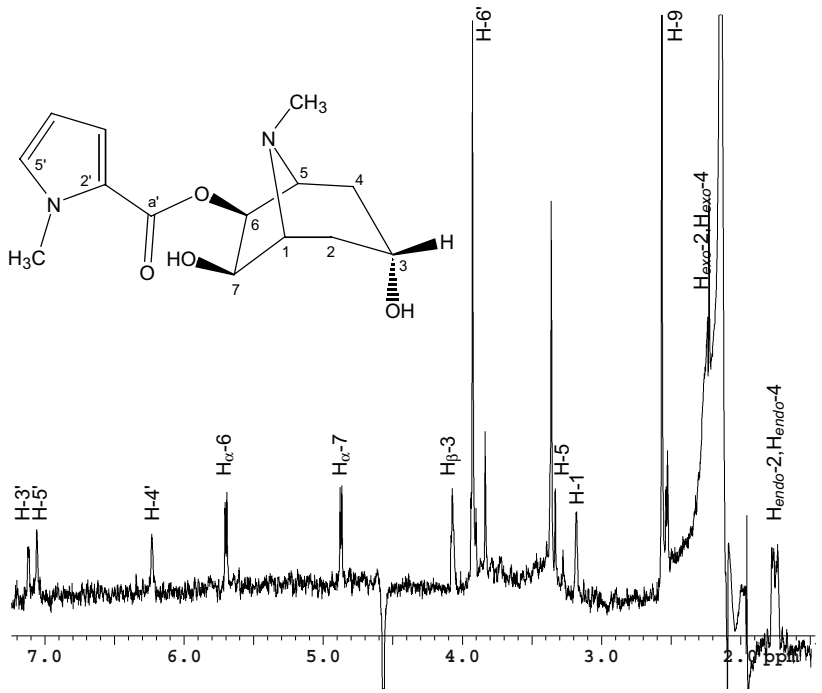


Figure 0-25

Stop-flow LC-^{1}H-NMR spectrum of compound **7** (RT = 14 min).

The stop-flow LC-^{1}H-NMR spectrum of compound **13** (RT = 20 min) showed similar chemical shifts to those of the spectrum of **7** supporting the presence in the molecule of a single methylpyrrole ester at C-6. The chromatographic peak at this retention time had a poor resolution, consequently, additional signals appeared in the spectrum (Figure 0-26). These extra signals probably belonged to compound **14**, a main alkaloid of the extract showing a large peak with a RT of 22 min. Subtracting the supplementary chemical shifts, the remaining signals were attributed to compound **13**. The LC-MS^n experiment of **13** showed a protonated molecule at 265 Da, suggesting the lack of an oxygen atom when compared to **7**. For this compound only one exchangeable proton was recorded confirming the presence of only one hydroxyl group. The NMR data of **13** exhibited a signal at δ_H 2.71, suggesting a saturated methylene moiety at the 7-position as for compounds **22** and **24**. Compound **13** was then elucidated as a 3,6-disubstituted tropane alkaloid corresponding to the structure 3α-hydroxy-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

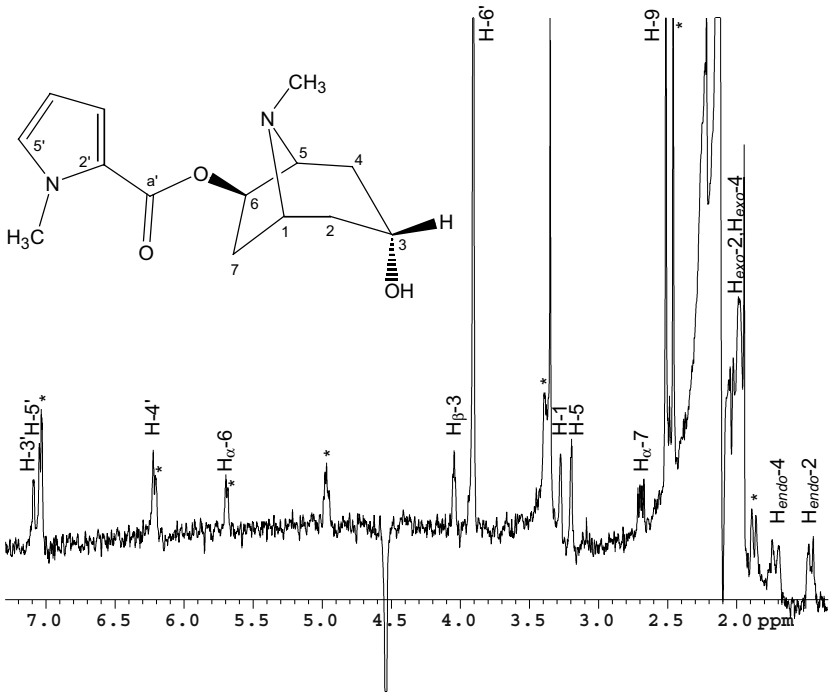


Figure 0-26

Stop-flow LC-^{1}H-NMR spectrum of compound **13** (RT = 20 min). (* are additional signals belonging to compound **14**)

From LC-^{1}H-NMR experiments, another main alkaloid (**14**) was detected (RT = 22 min). The on-flow LC-^{1}H-NMR indicated that **14** possessed a single methylpyrrole ester as in **7** and **13,** and the remaining signals supported the presence of a tropanic nucleus (Figure 0-27). Its LC-MS^n analyses exhibited $[M+H]^+$ and $[M_D+D]^+$ at 265 Da and 267 Da, respectively, demonstrating isomeric character with compound **13**. Despite a careful analysis of the NMR data only one deshielded tropanic proton was observed at δ_H 4.99 suggesting that the water suppression around 4.5 ppm affected the signal detection of the second oxygenated methine. The recorded values of the oxygenated methines at δ_H 4.99 and probably around 4.5 for **14** were different from those measured for the other compounds, which could implicate a variation in the oxygenated pattern of the tropanic skeleton. Accordingly, these variations impeded the complete structural elucidation of this compound.

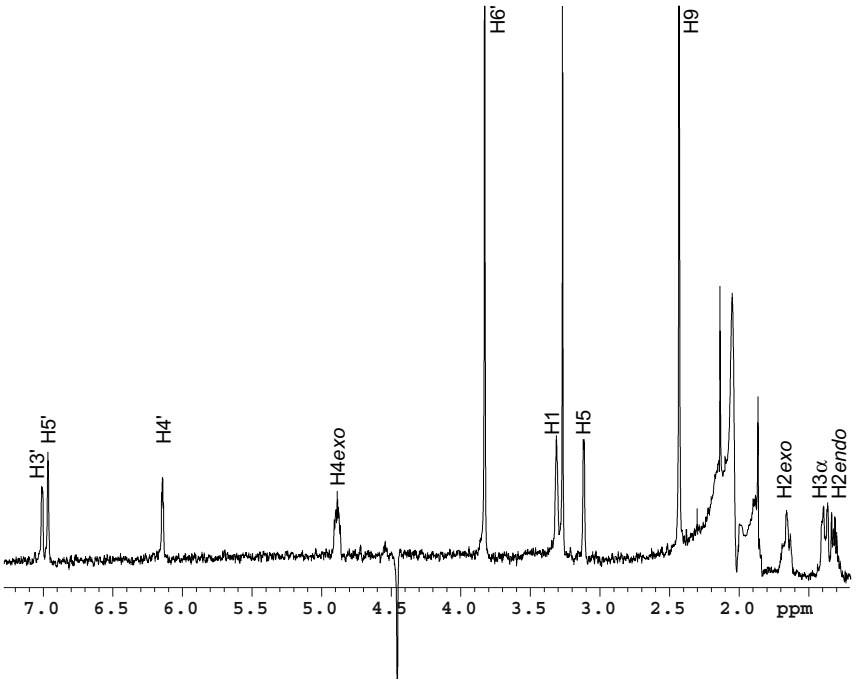


Figure 0-27

On-flow LC-^{1}H-NMR spectrum of compound **14** (RT = 22 min).

Partial identification of the minor alkaloids. The LC-MS^{n} analysis of the other constituents of the alkaloid extract showed similar patterns of fragmentation for each compound, giving important information for their core structures. The selective MS^{2} of each peak showed that all the different molecules shared a central structural element with fragment ions at *m/z* 140, 138, 122 or 120. Comparing this fragmentation pathway to that of the other on-line elucidated compounds and to the literature (Graf and Lude, 1978), these corresponded to a monohydroxylated tropane or a tropane moiety. The difference of 2 Da between fragments of the same type was related to the nature of their parent ions: disubstituted tropane alkaloids gave daughter ions at *m/z* 140 and 122, while trisubstituted tropane moieties gave daughter ions at *m/z* 138 and 120. Two other fragment ions with low *m/z* (110 and 108) were also found in almost all molecules and they probably corresponded to the ester moieties. Based on the assignments made for the previously elucidated alkaloids and on literature data (Graf and Lude, 1978), these fragments were identified as 1-methyl-2-(oxoniomethyl)-1*H*-pyrrolium groups (*e.g.*, see fragment structure of **23** in Figure 0-28). As illustrated by the MS^{3} experiments of ions at *m/z* 156 and 140, the fragments at *m/z* 110 and 108 could also be

obtained from the fragmentation of a tri- or dioxygenated tropane center. Due to its higher MW, compound **23** was selected as an example to illustrate a hypothetical fragmentation pathway for the tropane alkaloids of *E. vacciniifolium* (Figure 0-28).

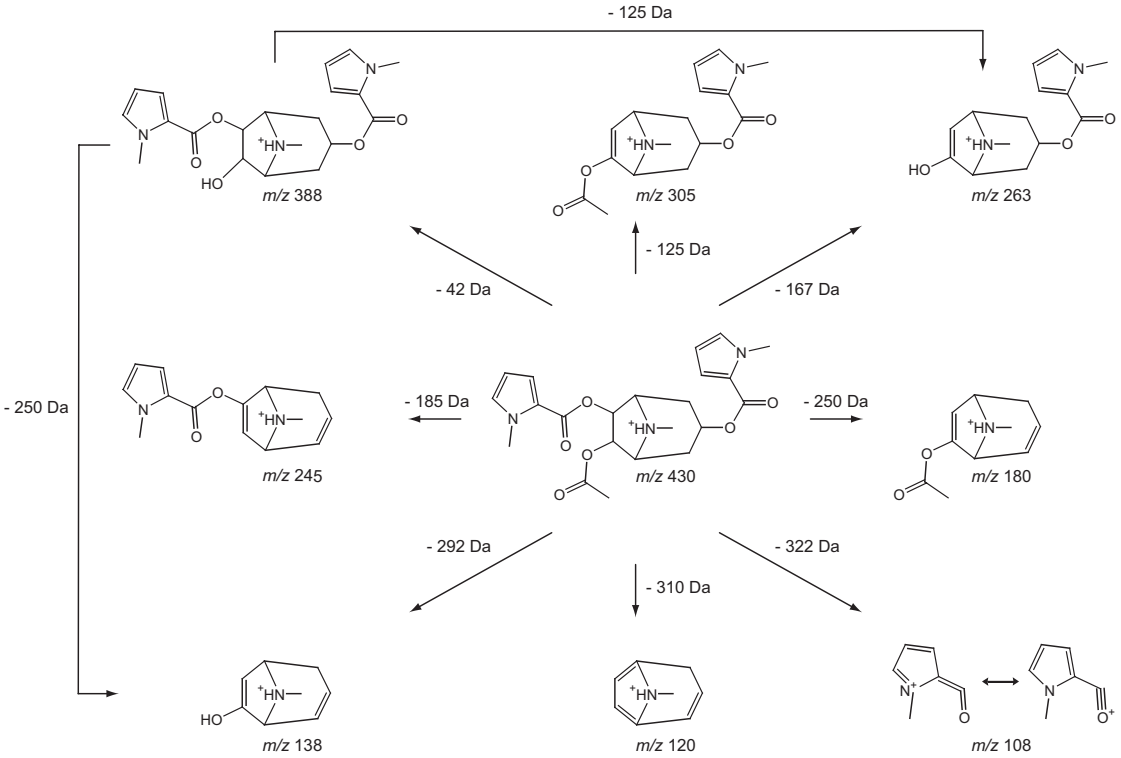


Figure 0-28

Proposed fragmentation pattern for protonated compound **23**. Due to the higher MW, compound **23** was selected as an example. The other molecules gave similar low MW fragment ions. MS^n conditions: capillary temperature: 150°C; vaporizer temperature: 380°C; corona needle current: 6.0 μA; sheath gas (nitrogen) pressure: 60 psi; positive mode; collision energy: 35%.

The superposition of the MS^2 and MS^3 traces at *m/z* 140, 138, 122, 120, 110 or 108 with the TIC trace of the extract showed that almost all detected molecules had the same daughter ions (Figure 0-29). This evaluation suggested that the differences among nearly all tropane molecules relied on the number and position of the ester substituents or hydroxyl groups. Blossey *et al*. reported the possibility of differentiating these positions in disubstituted tropane alkaloids by EI-MS, demonstrating the influence of functional groups on the fragmentation pattern of the tropane nucleus (Blossey *et al.*, 1964). In this study, the fragments obtained by collision induced dissociation in the LC-APCI-MS^n were not sufficiently homogeneous for a definitive determination of substitution positions.

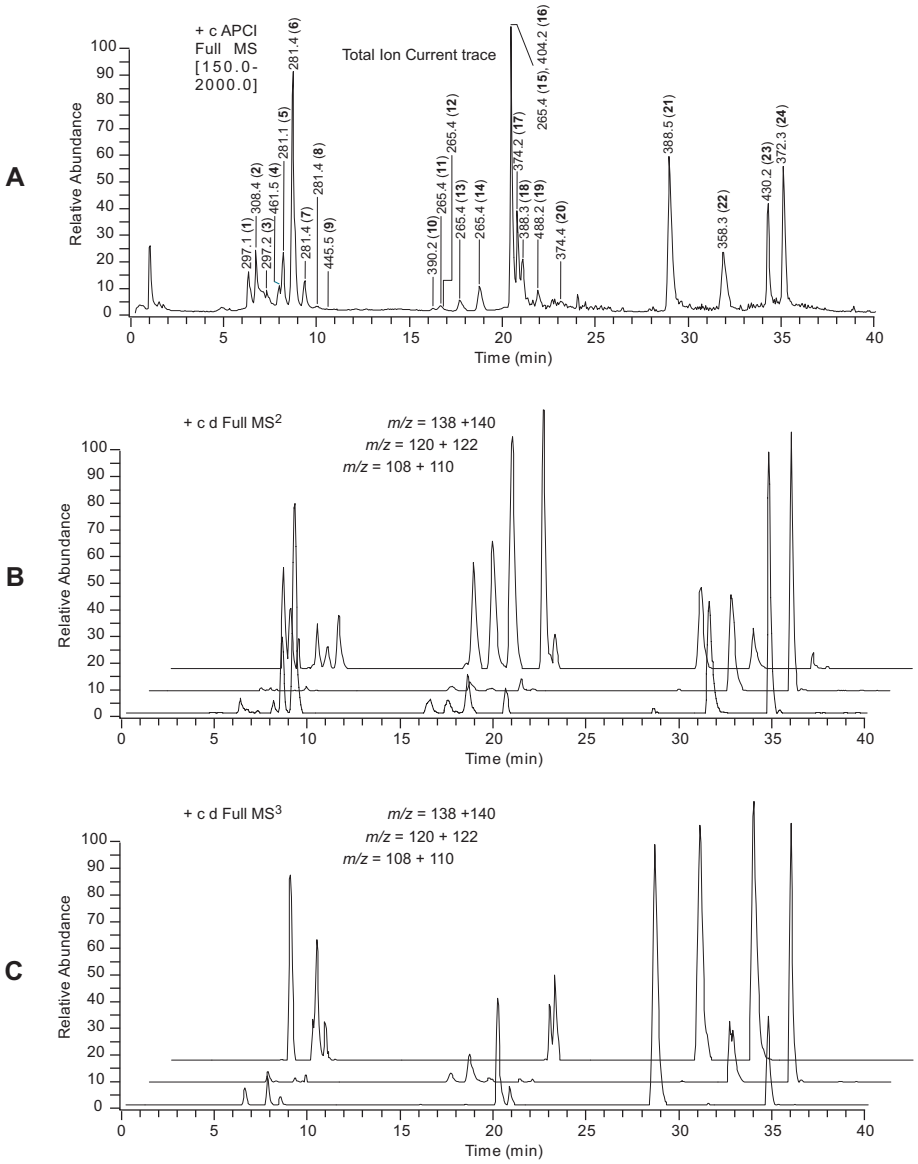


Figure 0-29

(**A**) LC-UV-APCI-MS analysis of the crude alkaloid extract of *E. vacciniifolium*. The total ion current (TIC) trace was recorded in the positive mode between 150 Da and 2000 Da. (**B**) Selective MS^2 traces of characteristic fragment ions. (**C**) Complementary MS^3 traces of low MW fragments. MS^n conditions: capillary temperature: 150°C; vaporizer temperature: 380°C; corona needle current: 6.0 μA; sheath gas (nitrogen) pressure: 60 psi; collision energy: 35%.

The combined interpretation of LC-UV-MS^n data led to the conclusion that all compounds with an absorbance maximum around 270 nm were esterified by one or two pyrrolcarboxylic acids or contain one or two pyrrolic chromophores, as for **7**, **13** and **21**-**24** identified on-line. Comparison of these data for the minor alkaloids with those previously elucidated gave important information for their characterization. Most of these alkaloids could be grouped in sets of isomeric structure. In many cases a molecular weight difference of 16 Da between these sets were recorded, indicating variation due to additional oxygen. The extra oxygen atoms could also appear in their fragment ions (*e.g. m/z* 263 and 247 for **21** and **24** in Table 0-5). This molecular ion difference occurred between compounds: **5**-**8** (each 281 Da) and **1**, **3** (each 297 Da); **9** (445 Da) and **4** (461 Da); **11**-**15** (each 265 Da) and **5**-**8** (each 281 Da); **17**, **20** (each 374 Da) and **10** (390 Da); **22** (358 Da) and **17**, **20** (each 374 Da); **24** (372 Da) and **18**, **21** (each 388 Da). In order to confirm that these oxygens corresponded to an additional hydroxyl group in the molecules, the number of exchangeable protons was calculated as mentioned before. Compounds **17** and **20** exhibited the same protonated molecule (16 Da more than **22**), but they had different numbers of exchangeable protons (1 for **17** and 2 for **20**). This observation suggested an additional hydroxyl group for **20** and probably the presence of an *N*-oxide in compound **17**. A similar deduction could also be made for compounds **18** and **21**. They had the same $[M+H]^+$ (16 Da more than **24**) but only **21** showed an additional exchangeable proton, suggesting the presence of an extra hydroxyl group. Therefore, compound **18** probably included an *N*-oxide in its structure (Figure 0-30). In the same way, five other tropane alkaloids (**1**, **3**, **5**, **10** and **16**) were deduced to contain an *N*-oxide in their structure in comparison with **7**, **8**, **13**, **20** and **21**, respectively. The occurrence of tropane alkaloid *N*-oxides in plants has previously been reported (Aripova, 1996; Katavic *et al.*, 1999; Phillipson and Handa, 1978; Silva *et al.*, 2001). The ratio of *N*-oxides to tertiary bases in plants varies for different organs during ontogenesis. This fluctuation is an indication that *N*-oxides are not artifacts but that they are involved in the metabolic processes of the plant (Phillipson and Handa, 1978). In contrast, Achenbach *et al.* reported that model experiments performed with indole alkaloids showed *N*-oxidation occurring in presence of air or during work-up of plant extracts (Achenbach *et al.*, 1997). These divergent considerations do not allow to conclude whether *N*-oxides are genuine natural products or artifacts.

Compound **19** did not present any exchangeable protons and seemed to share a similar core structure with **18**. In fact, its most abundant fragment obtained by MS^2 (*m/z* 388) corresponded to the protonated molecule of **18**. After a comparison with the literature on tropane alkaloids, this fragment was assigned to the loss (*m/z* 100) of an isovaleranyl moiety (Christen *et al.*, 1993).

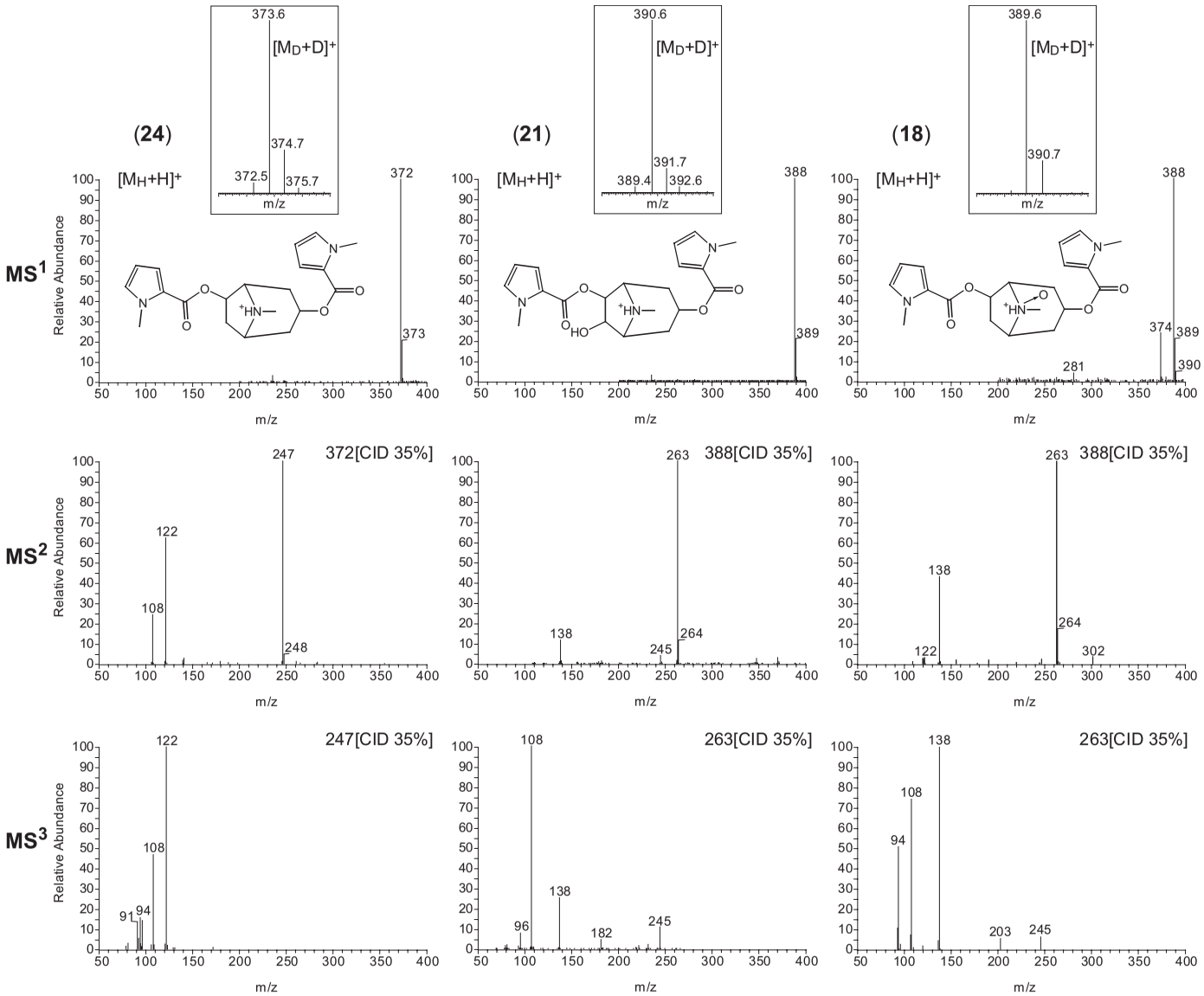


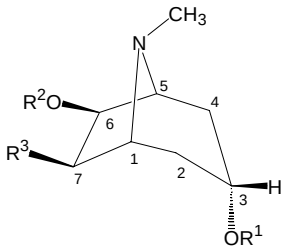
Figure 0-30

MS^n spectra of compounds **24**, **21** and **18**. The two latter compounds have the same $[M+H]^+$ (16 Da more than **24**) but only **21** showed an additional exchangeable proton.

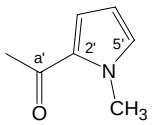
Compounds **9** and **4** had three absorbance maxima (241, 271 and 329 nm), indicating the presence of at least one different substituent. The MS^2 analysis of these two compounds gave typical fragment ions at *m/z* 247 and 263, respectively, for a tropane or a hydroxytropane esterified by a methylpyrrole, but a new daughter ion at *m/z* 181 appeared in the fragmentation pathway, confirming the presence of a new esterifying group. According to the fragmentation pattern of catuabine A, previously isolated from *E. vacciniifolium* (Graf and Lude, 1978), this fragment ion probably corresponded to a [(4-hydroxy-3,5-dimethoxyphenyl)methylidyne]oxonium. The presence of this ester was confirmed by the MS^3 experiment of **9** which gave fragment ions at *m/z* 181, 140 and 122 from a parent ion at *m/z* 320 assigned to a tropane moiety esterified by 4-hydroxy-3,5-dimethoxybenzoic acid. The trimethoxybenzoic acid ester in tropane alkaloids is relatively common and its occurrence was easily detected by MS-MS experiments giving the typical fragment at *m/z* 195 for its acyl unit (Agar and Evans, 1976; Bringmann *et al.*, 2000a; El-Iman *et al.*, 1987; Silva *et al.*, 2001). The presence of an additional hydroxyl group in **4** was also confirmed by the observation of one more exchangeable proton than in **9**.

The presence in the extract of another tropane alkaloid with an original substituent was demonstrated by the occurrence of a different chromophore for compound **2** (two maxima of absorbance at ca. 241 and 294 nm). The absence of a maximum around 270 nm suggested the lack of a pyrrolic chromophore. The MS^2 experiments of **2** gave typical fragment ions for a tropane center with a most abundant ion at *m/z* 156, corresponding to a dihydroxylated moiety. The loss of the substituent during the fragmentation was shown by the loss of 152 Da from the molecular ion (308 Da) of **2**. According to the literature on tropane alkaloids (Silva *et al.*, 2001), this esterifying group could be a 3-hydroxyphenylacetic acid, but supplementary analysis is needed for unambiguous identification.

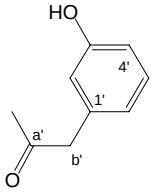
On the basis of the above interpretation of the LC-UV-MS^n spectroscopic data and by analogy with the on-line elucidated compounds, **1**-**6**, **8**-**12** and **14**-**20** were partially identified as di- or trisubstituted tropane alkaloids, mono- or diesterified by pyrrolic, methylpyrrolic, hydroxydimethoxybenzoic or hydroxyphenylacetic acids (Figure 0-31). The pyrrole substituent is uncommon in tropane alkaloids (only three have been isolated previously) and may play an important role in the biological activity of the compounds. The proposed structures of these compounds suggested them to be new and their targeted isolation was undertaken for complete characterization.



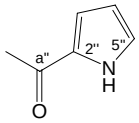
Mpc =



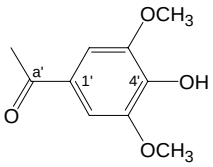
3-OHPhCH$_2$CO =



Pc =



Hdmb =



Compound[a]	R^1	R^2	R^3	Other
7	H	Mpc	OH	-
13	H	Mpc	H	-
21	Mpc	Mpc	OH	-
22	Mpc	Pc	H	-
23	Mpc	Mpc	CH_3COO	-
24	Mpc	Mpc	H	-
Compound[b]	Mpc	Pc	OH	Other
1	1	-	2	N→O
2	-	-	2	3-OHPhCH$_2$CO
3	1	-	2	N→O
4	1	-	1	Hdmb
5	1	-	1	N→O
6	1	-	2	-
8	1	-	2	-
9	1	-	-	Hdmb
10	1	1	1	N→O
11	1	-	1	-
12	1	-	1	-
14	1	-	1	-
15	1	-	1	-
16	2	-	1	N→O
17	1	1	-	N→O
18	2	-	-	N→O
19	2	-	-	N→O; C_4H_9COO
20	1	1	1	-

[a] Fully identified and characterized compounds.
[b] Partially identified compounds.

Figure 0-31

Structures of tropane alkaloids from *E. vacciniifolium* identified on-line. All compounds had a di- or trisubstituted tropane moiety mono- or diesterified by Mpc, Pc, Hdmb, 3-OHPhCH$_2$CO or C_4H_9COO. Several tropane centers had an *N*-oxide group. The structures differed in the number and position of the ester substituents or hydroxyl groups.

2.3. Fractionation and purification of the alkaloid extract by MPLC and semi-preparative HPLC

The alkaloid extract (10 g) was fractionated by MPLC with MeCN-H_2O-2 mM Et_3N (460 x 70 mm, flow rate: 5.0 mL/min, gradient: MeCN 5% to 100% in 3 days, UV detection at 280 nm) to give 13 fractions (A to M). Fraction L yielded directly in one step compound **22** (813 mg). The scheme of fractionation and isolation of all compounds is reported in Figure 0-32.

Fraction F was rechromatographed by MPLC with MeCN-H_2O-2 mM Et_3N (460 x 36 mm, flow rate: 3.4 mL/min, gradient: MeCN 5% to 15% in 24 hs, UV detection at 280 nm) to give 5 fractions (F1 to F5). Fractions F2 and F5 yielded compounds **2** (180 mg) and **8** (117 mg), respectively.

Fraction G was purified by MPLC MeCN-H_2O-2 mM Et_3N (460 x 36 mm, flow rate: 6.6 mL/min, gradient: MeCN 5% to 10% in 8 hs, UV detection at 280 nm) to afford compound **7** (198 mg).

Fraction H was separated by MPLC with MeCN-H_2O-2 mM Et_3N (460 x 36 mm, flow rate: 3.4 mL/min, gradient: MeCN 5% to 30% in 47 hs, UV detection at 280 nm) to give 10 fractions (H1 to H10). Fractions H7 and H10 yielded compounds **11** (17 mg) and **15** (86 mg), respectively.

Fraction I was purified by MPLC MeCN-H_2O-2 mM Et_3N (460 x 36 mm, flow rate: 5.0 mL/min, gradient: MeCN 5% to 20% in 20 hs, UV detection at 280 nm) to afford compound **14** (543 mg).

Fraction J was purified by MPLC with MeCN-H_2O-2 mM Et_3N (460 x 36 mm, flow rate: 3.0 mL/min, gradient: MeCN 5% to 25% in 2.5 days, UV detection at 280 nm) to give 8 fractions (J1 to J8). Fractions J1, J3, J6 and J7 yielded respectively compounds **4** (13 mg), **9** (33 mg), **18** (37 mg) and **20** (8 mg). Fraction J4 was rechromatographed by HPLC with MeCN-H_2O-2 mM Et_3N (Nucleosil 100-5 C_{18} AB, 125 x 8 mm, 5 µm, flow rate: 2.0 mL/min, isocratic: MeCN 13% for 30 min, UV detection at 272 nm) to afford compounds **13** (22 mg) and **12** (7 mg).

Fraction K was separated by MPLC with MeCN-H_2O-2 mM Et_3N (460 x 36 mm, flow rate: 5.0 mL/min, gradient: MeCN 15% to 60% in 4 hs, UV detection at 280 nm) to give 9 fractions (K1 to K9). Fraction K3 yielded compound **6** (81 mg), while fractions K6 and K8 gave compound **21** (631 mg) and compound **23** (232 mg), in that order.

Fraction M was purified by MPLC MeCN-H_2O-2 mM Et_3N (460 x 36 mm, flow rate: 5.0 mL/min, gradient: MeCN 15% to 60% in 19 hs, UV detection at 280 nm) to afford compound **24** (727 mg).

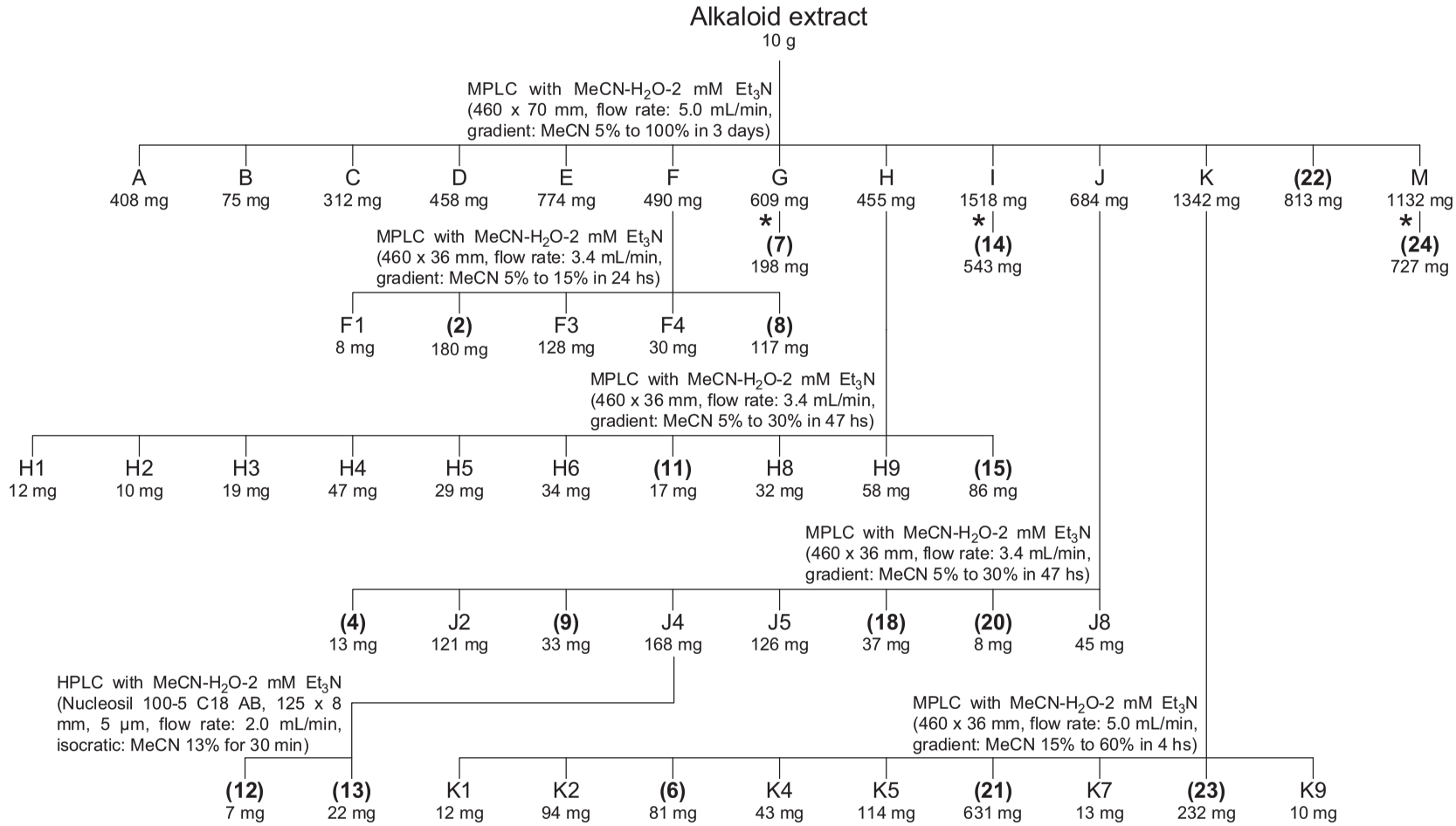


Figure 0-32

Isolation scheme for compounds **2**, **4**, **6**-**9**, **11**-**15**, **18** and **20**-**24**. (* purification by MPLC with MeCN-H_2O-2 mM Et_3N)

2.4. Structure elucidation of catuabines D to I, vaccinines A and B, and their derivatives

2.4.1. Catuabine D

Catuabine D (**22**) was isolated as white amorphous powder. High-resolution electrospray ion cyclotron resonance mass spectroscopic analysis (HRESMS) of this compound suggested a molecular formula of $C_{19}H_{23}N_3O_4$, implicating 10 centers of unsaturation and/or ring structures. The ^{13}C-NMR spectrum recorded in chloroform-*d* indicated 10 sp^2-hybridized carbon atoms (Table 0-11; Figure 0-33), of which six had protons attached and four were non-protonated. The IR spectrum of **22** exhibited a large absorption band at 1700 cm^{-1} indicating the presence of ester group(s) (Figure 0-34).

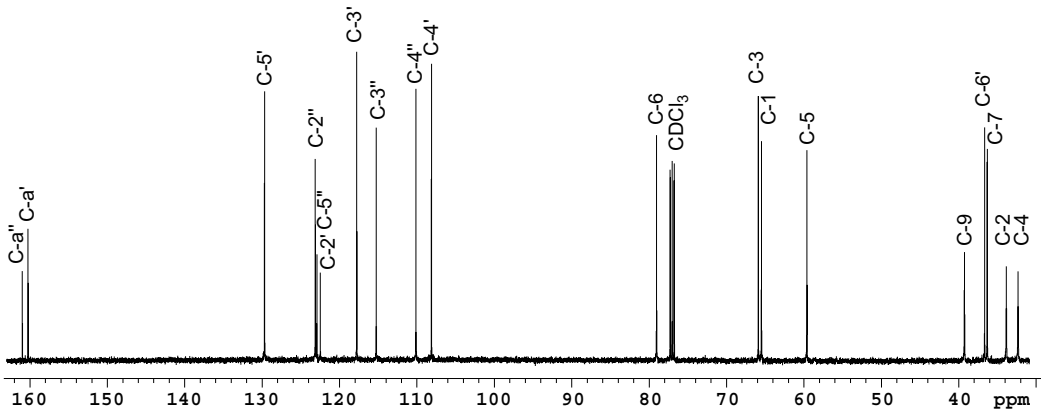


Figure 0-33

^{13}C-NMR spectrum of compound **9**. The ^{13}C assignments were ascertained by DEPT, gHSQC, and gHMBC experiments. (Spectrum recorded in $CDCl_3$ at 125.70 MHz using $CDCl_3$ as internal standard)

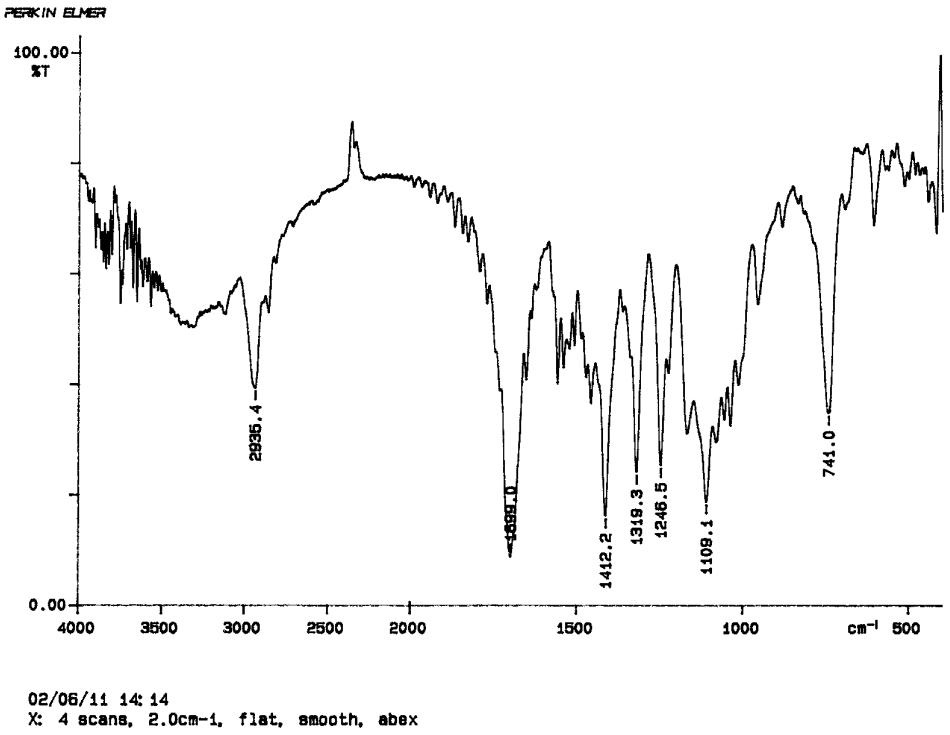


Figure 0-34

IR spectrum of compound **22**. (KBr pellet; 4 scans; 2.0 cm^{-1}; 500-4000 cm^{-1})

According to the number of sp^2-hybridized carbons, two ester functions associated with chemical shifts at δ_C 160.9 and 160.3 and four ethylenic groups were required. These six centers of unsaturation suggested consequently the presence of four rings in the molecule. A gDQF-COSY NMR experiment on **22** indicated four isolated spin systems corresponding to the four different rings (Figure 0-35).

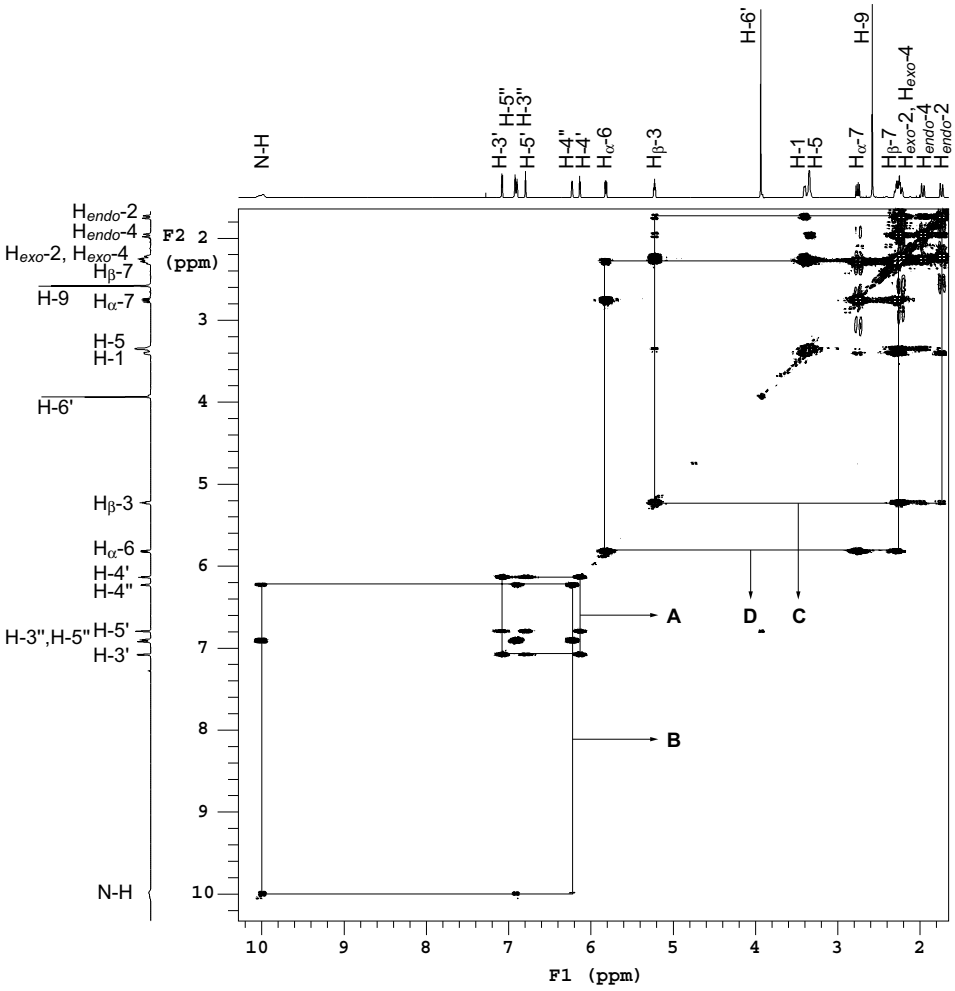


Figure 0-35

gDQF-COSY spectrum of compound **22** showing four isolated spin systems (**A**: methylpyrrole ring; **B**: pyrrole ring; **C**: piperidine ring; **D**: pyrrolidine ring)

The ^{1}H-NMR spectrum of one of these spin systems exhibited typical resonances for a methylpyrrole substructure with signals at δ_H 3.94 (N-CH_3), δ_H 6.15 (H-4'), δ_H 6.80 (H-5') and δ_H 7.08 (H-3'), respectively (Figure 0-36). The gHMBC spectrum showed characteristic long-range ^{1}H-^{13}C correlations between the methyl group at δ_H 3.94 (N-CH_3) and two carbons of the pyrrole ring at δ_C 122.5 (C-2') and 129.7 (C-5'). In addition, the gHMBC spectrum exhibited a correlation between the pyrrole proton at δ_H 7.08 (H-3') and the carbonyl carbon at δ_C 160.3 (C-a') indicating then the substitution of the methylpyrrole moiety at the 2'-position by an ester group (Figure 0-37).

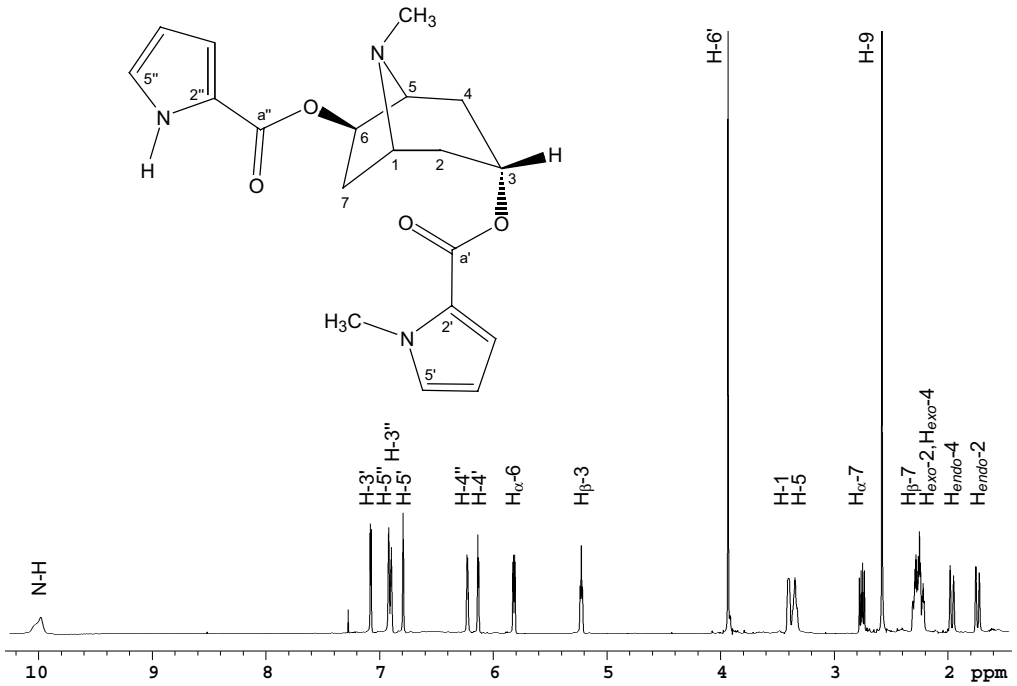


Figure 0-36

^{1}H-NMR spectrum of compound **22**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

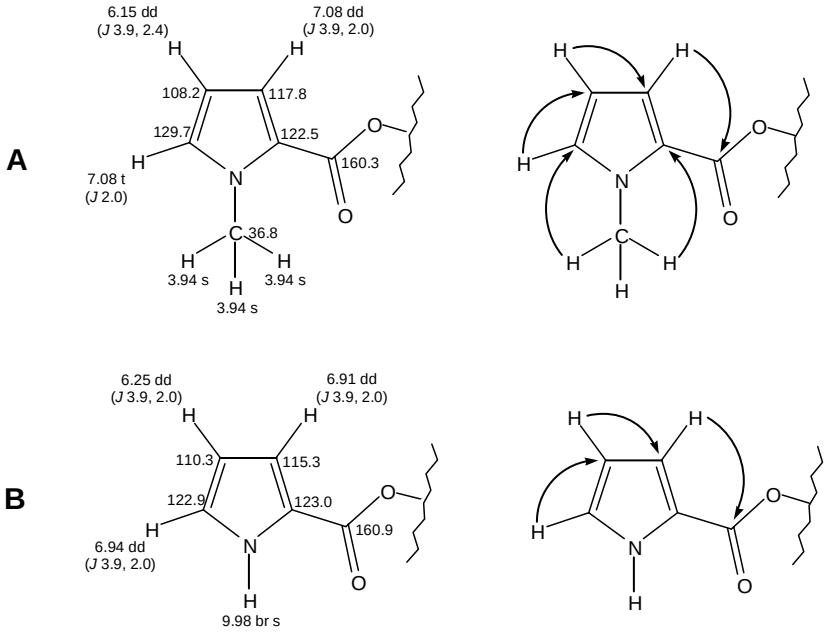


Figure 0-37

Characteristic ^{1}H and ^{13}C-NMR data for a methylpyrrole ester (spin system **A**) and a pyrrole ester (spin system **B**), and their most important long-range ^{1}H- ^{13}C correlations. (δ values given in ppm; *J* values in parentheses given in Hz)

Another spin system deduced from the gDQF-COSY experiment was associated with a second pyrrole substructure characterized by resonance signals at δ_H 6.25 (H-4''), δ_H 6.94 (H-5''), δ_H 6.91 (H-3'') and δ_H 9.98 (H-N), respectively. Deduced from the observation of a long-range ^1H-^{13}C correlation between H-3'' and the carbonyl carbon at δ_C 160.9 (C-a'') by a gHMBC experiment on **22**, the 2-position of this second pyrrole substructure was also substituted by an ester moiety (Figure 0-37).

The two other remaining spin systems belonged to the tropane alkaloid skeleton. The ^1H-NMR spectrum of **22** exhibited a singlet signal at δ_H 2.58 attributed to the protons of a methyl group bonded to the nitrogen (Figure 0-36). The gHMBC experiment showed ^1H-^{13}C correlations between these methyl protons and two methine moieties at δ_C 65.5 and 59.6 ppm corresponding to C-1 and C-5, respectively (Figure 0-38; Figure 0-39). According to the gHSQC spectrum, these two methine carbons were linked to protons with resonances at δ_H 3.40 (H-1) and δ_H 3.34 (H-5) (Figure 0-40). The gDQF-COSY experiment showed that the two methine protons belonged to a first spin system of five protons arranged in a 2-oxopropyl substructure with signals at δ_H 1.74 (H_{endo}-2), 1.96 (H_{endo}-4), 2.22 (H_{exo}-2), 2.25 (H_{exo}-4) and 5.23 (H-3β). In addition, the methine proton H-1 was linked to a second spin system of three coupled protons characterized by chemical shifts at δ_H 5.81 (H-6α), 2.28 (H-7β) and 2.76 (H-7α) (Figure 0-35).

The exact substitution of the tropane alkaloid skeleton was then confirmed by examination of gHMBC spectra: the resonance at δ_H 5.81 (H-6α) showed a long-range ^1H-^{13}C correlation with a methylene group at δ_C 32.2 (C-4), and the signals at δ_H 2.28 (H-7β) and δ_H 2.76 (H-7α) correlated with the carbon at δ_C 33.7 (C-2). To conclude, the long-range ^1H-^{13}C correlations between the H-3 and H-6 protons and the carbonyl carbons C-a' and C-a'' of the two pyrrole substructures, respectively, led to the general structure 3-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-6-[(1*H*-pyrrol-2-yl)carbonyloxy]tropane.

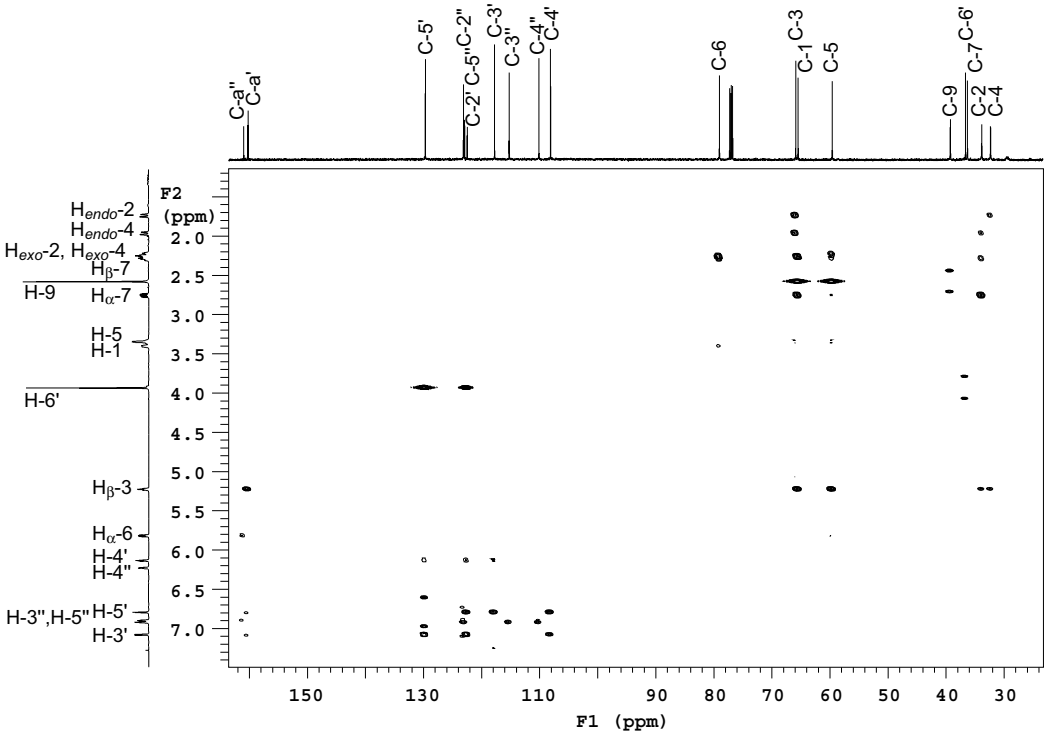
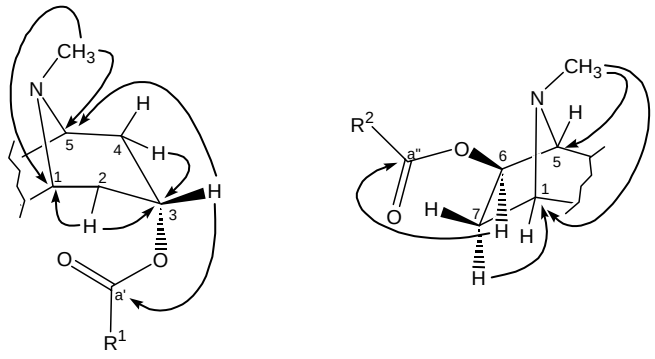


Figure 0-38

gHMBC spectrum of compound **22**.



C **D**

Figure 0-39

Selected long-range ^{1}H- ^{13}C correlations of tropane moiety (spin systems **C** and **D**).

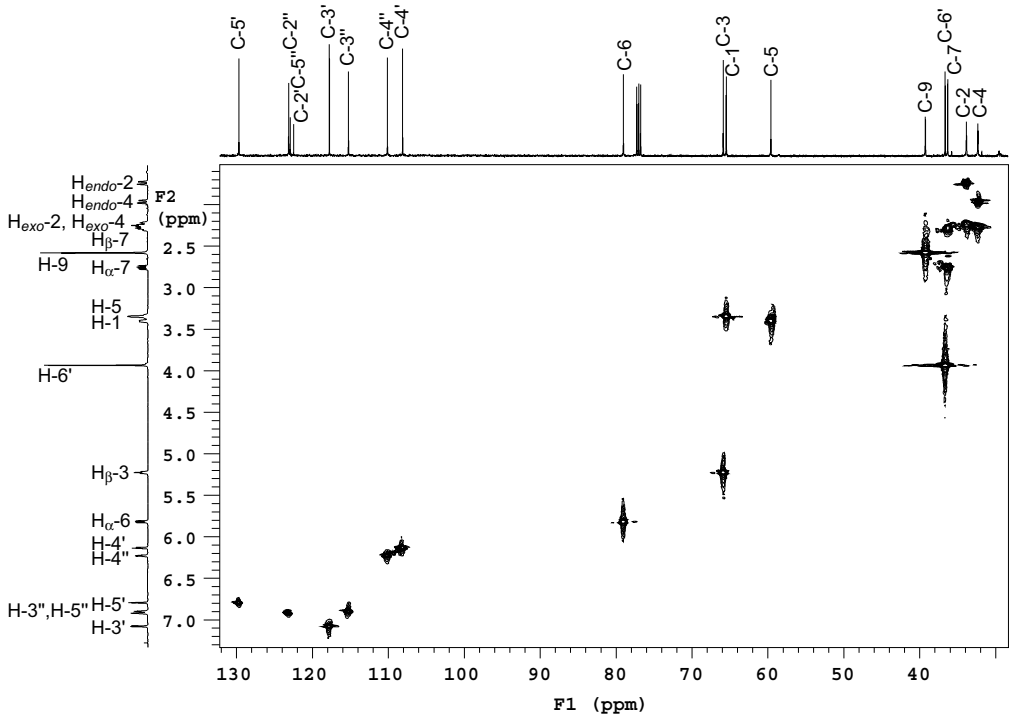


Figure 0-40

gHSQC spectrum of compound **22**.

The relative configuration of **22** was established in order to give the stereochemical orientation of the two substituents relative to the nitrogen-containing bridge (Figure 0-41). The multiplicity (triplet) of the H-3 signal with the coupling constant (J = 4.9 Hz) indicated the α-orientation (*i.e. endo*) of the substituent at C-3 (Agar and Evans, 1976; Al-Said *et al.*, 1989a; El-Iman *et al.*, 1987). The arrangement of the substituent at C-6 was established by the analysis of the coupling constants of H-6, H-7 and H-5 protons. The H-6 proton showed two couplings (7.3, 2.9) with the two H-7 protons and it did not present any coupling with the vicinal H-5 proton. This observation implied a β-orientation of the substituent and a dihedral angle close to 90° between H-5 and H-6α (Al-Said *et al.*, 1986a; Bringmann *et al.*, 2000a). The tropane N-CH_3 group stereochemistry was established by NOESY NMR experiments. The clear NOESY interactions between the N-CH_3 group and the H_{exo}-2 and H_{exo}-4 indicated an axial orientation of the N-CH_3 group in the molecule.

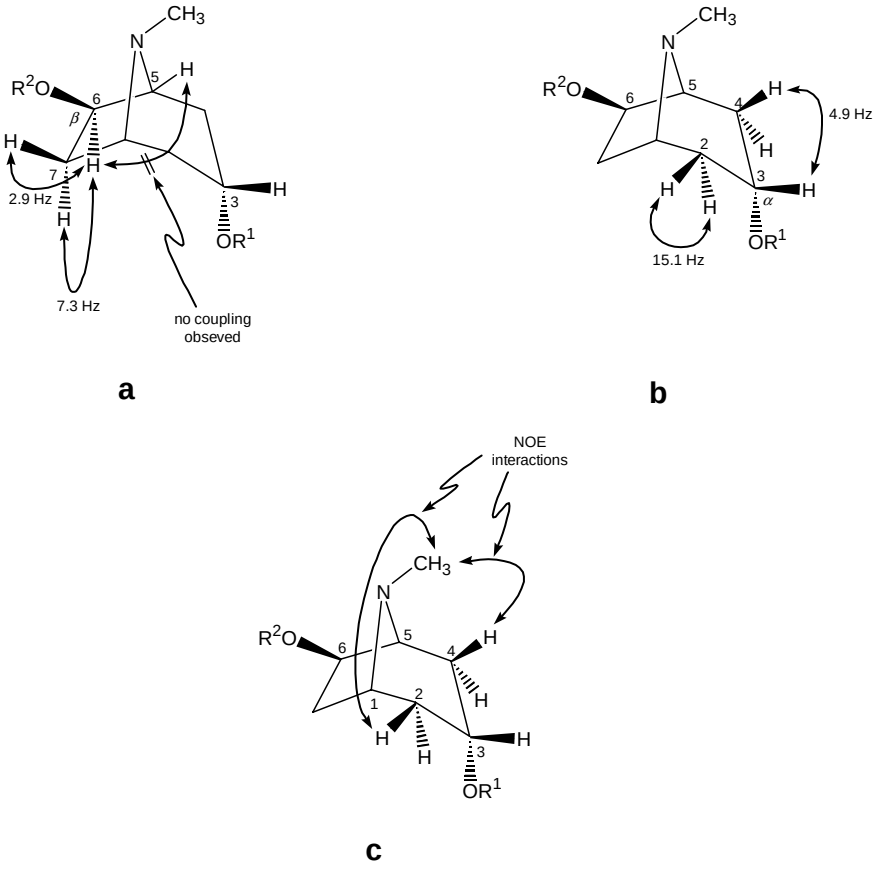


Figure 0-41

Relative configuration of compound **22**, as deduced from ^{1}H-NMR coupling constants (**a** and **b**) as well as NOESY interactions (**c**).

Thus, the structure of catuabine D (**22**) is 3α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-6β-[(1*H*-pyrrol-2-yl)carbonyloxy]tropane.

2.4.2. 7β-Hydroxycatuabine D

Compound **20** was purified as white amorphous powder and was assigned a molecular formula of $C_{19}H_{23}N_3O_5$, as determined by HRESMS. The EI mass spectrum showed a molecular ion at *m/z* 373, 16 amu higher than that of **22**, suggesting the occurrence of an additional oxygen atom in the structure of alkaloid **20**. The NMR data of those two compounds were also closely related, indicating the presence in **20** of a central tropane moiety esterified by two methylpyrrole (Mpc) or pyrrole (Pc) acids. By the analysis of the gHMBC spectrum, the methylpyrrole and pyrrole esters were located, as for catuabine D, at positions

C-3 and C-6, respectively. Except for proton H-7, the ^{1}H-NMR spectrum of the tropane nucleus of **20** exhibited similar chemical shifts to **22** (Table 0-10; Figure 0-42). Actually, this spectrum showed typical resonances for a tropane alkaloid skeleton trisubstituted at C-3, C-6 and also C-7 positions with signals at δ_H 5.27 (H-3β), δ_H 5.80 (H-6α) and δ_H 4.88 (H-7α), respectively. According to the molecular formula and the values of the chemical shifts associated with the 7-position (δ_H 4.88 and δ_C 74.6), a hydroxyl moiety was required at this location.

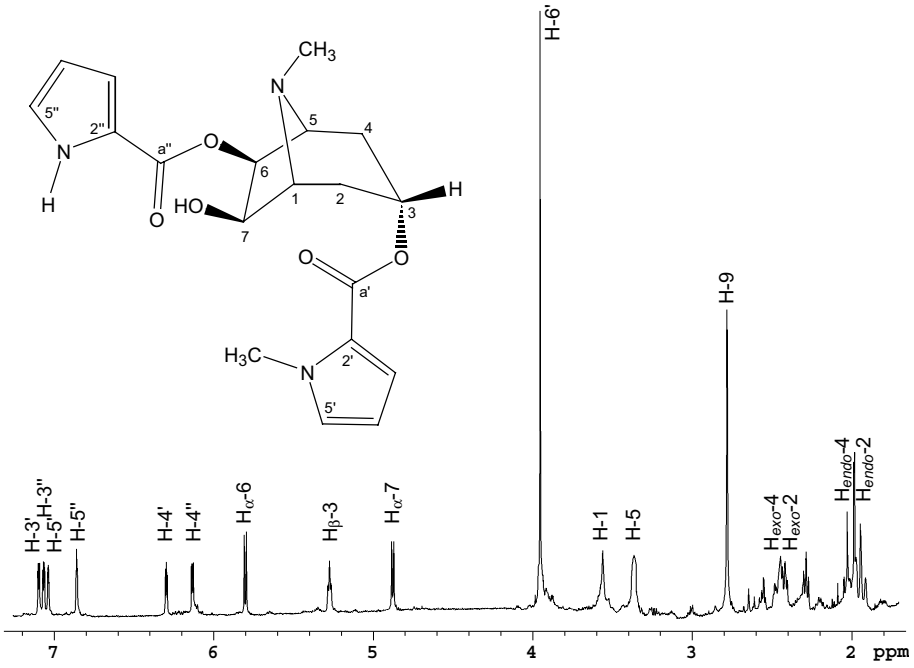


Figure 0-42

^{1}H-NMR spectrum of compound **20**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

The α-orientation of the esterifying group in C-3 was resolved from the multiplicity (triplet) and the coupling constant (J = 4.9 Hz) of the H-3 proton signal (Agar and Evans, 1976; Al-Said *et al.*, 1989b; El-Iman *et al.*, 1987). The α-orientation of protons at C-6 and C-7 position was deduced by the lack of any coupling constant with vicinal protons H-5 and H-1, respectively.

Hence, the structure of **20** (7β-hydroxycatuabine D) was deduced as 3α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-6β-[(1*H*-pyrrol-2-yl)carbonyloxy]-7β-hydroxytropane.

2.4.3. Catuabine E

Alkaloid **24** was purified as white amorphous powder and also exhibited spectroscopic data similar to those of **22**. The molecular formula was obtained as $C_{20}H_{25}N_3O_4$. The EI mass spectrum showed a molecular ion 14 amu higher than that of **22** and a similar fragmentation pattern, suggesting the presence of an additional methyl group in the structure of compound **24**. The NMR data strongly resembled those of **22**, consistent with a general structure containing a central tropane moiety dioxygenated at C-3 and C-6, and esterified by two methylpyrrole acids. The signal integrations of the ^{1}H-NMR spectrum confirmed the presence of two methyl groups, one at δ_H 3.93 (3H, s, N-CH_3) and the other at δ_H 3.94 (3H, s, N-CH_3), linked to the nitrogen atoms of the two pyrrole rings (Figure 0-43). Analysis of the gDQF-COSY spectrum showed two distinct spin systems for the substituents, allowing assignment of each proton to the corresponding methylpyrrole ester and positioning of the two Mpc at positions C-3 and C-6, according to the gHMBC experiment.

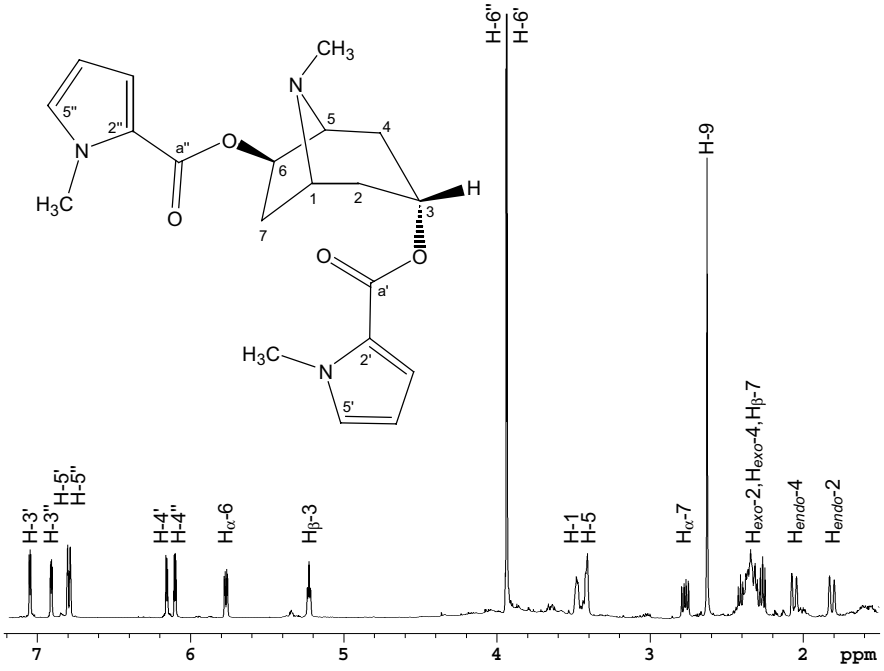


Figure 0-43

^{1}H-NMR spectrum of compound **24**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

The relative configuration of **24** was identical to that of **22**.

On the basis of the above evidence, the structure of **24** (catuabine E) was elucidated as 3α,6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

2.4.4. 7β-Hydroxycatuabine E

Compound **21** was isolated as a white amorphous powder and gave a molecular formula of $C_{20}H_{25}N_3O_5$, according to a HRESMS experiment. Analysis of the NMR data of **21** indicated strong similarity to **20** and **24**. The ^{1}H-NMR spectrum of the tropane moiety exhibited the same pattern of substitution as that of **20**, with signals at δ_H 5.24 (H-3β), δ_H 5.71 (H-6α) and δ_H 4.79 (H-7α) (Figure 0-44). The other part of ^{1}H-NMR spectrum describing the acyl moieties was closely comparable to equivalent data of alkaloid **24** and indicated the presence of two Mpc groups as substituents. The positions of their attachment were determined by analyzing gDQF-COSY and gHMBC spectra as for compound **24**.

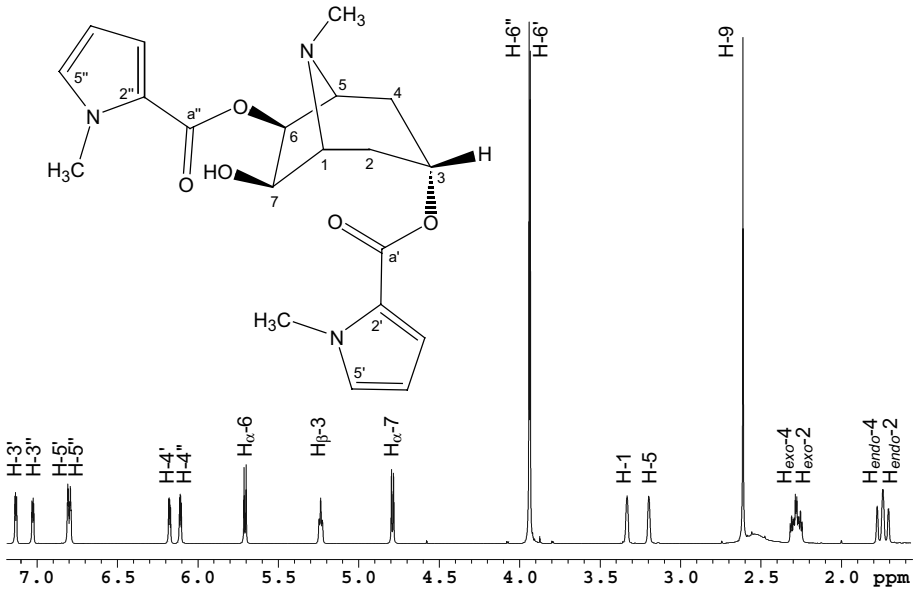


Figure 0-44

^{1}H-NMR spectrum of compound **21**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

The relative configuration of **21** was the same as that for **20**. The triplet for the proton at C-3 (J = 4.9 Hz) and the doublets for the protons at C-6 and C-7 (each J = 5.9 Hz) indicated α-, β- and β-orientations of the substituents, respectively.

Thus, alkaloid **21** was 3α,6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-7β-hydroxytropane, named 7β-hydroxycatuabine E.

2.4.5. 7β-Acetylcatuabine E

Alkaloid **23** was isolated as a white amorphous powder. The HRESMS analysis implied a molecular formula of $C_{22}H_{27}N_3O_6$ and the EIMS experiment showed a $[M]^+$ molecular ion at *m/z* 429. Similarity of fragmentation pattern and a molecular mass of 42 amu higher than that of **21** suggested a similar core structure to 7β-hydroxycatuabine E with an additional acetyl group. Analyses of the NMR data indicated a close resemblance of the structure of **23** to that of compound **21**. The presence of two Mpc moieties was established after analysis of the ^{1}H-NMR spectrum, which was superimposable with **21** between δ_H 6.0 and 7.3 (Figure 0-45). The assignment of each proton to the respective spin system was achieved by a gDQF-COSY experiment and the linkage of the esters to the tropane moiety was established by analyzing the gHMBC spectrum, as for the other compounds. The remainder of the ^{1}H-NMR spectrum showed typical chemical shifts for a trisubstituted tropane skeleton with resonances at δ_H 5.28 (H-3β), δ_H 5.88 (H-6α) and δ_H 5.81 (H-7α). The signal at δ_H 5.81, typically shifted downfield with respect to non-esterified compounds, like **20** or **21**, indicating esterification at C-7 (δ_C 77.7) rather than a free alcohol (Al-Said *et al.*, 1986a; El-Iman *et al.*, 1987; Payo-Hill *et al.*, 2000). The substituent at C-7 was shown to be an *O*-acetyl group by the signals at δ_H 2.04 (3H, s, H-b'''), δ_C 170.2 (C-a''') and δ_C 20.8 (C-b'''). Its position was confirmed by a gHMBC correlation between H-7 and C-a'''.

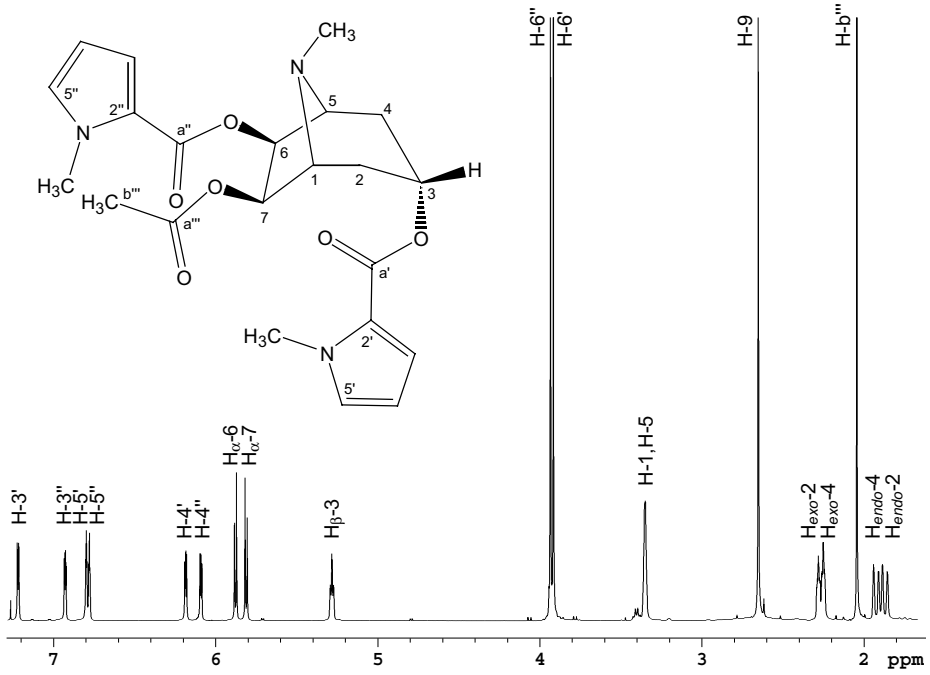


Figure 0-45

^{1}H-NMR spectrum of compound **23**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

The relative configuration of **23** was the same as for the other two trioxygenated tropane alkaloids **20** and **21**.

Thus, compound **23** was elucidated as 7β-acetoxy-3α,6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane and subsequently named 7β-acetylcatuabine E.

2.4.6. Catuabine E *N*-oxide

Compound **18** was purified as an amorphous solid. HRESMS analysis of its pseudomolecular ion $[M+H]^+$ indicated the molecular formula as $C_{20}H_{26}N_3O_5$. The analysis of NMR data demonstrated that compound **18** was a dioxygenated tropane substituted by two methyl pyrrole esters. The proton sequences and the carbon atom locations, respectively deduced by gDQF-COSY and gHMBC experiments, implicated the 3- and 6-positions for the two ester moieties of the tropane alkaloid. When compared to the previously described catuabine E (**24**), compound **18** seemed to have an additional oxygen atom (16 amu excess of molecular weight). Several chemical shifts recorded in ^{1}H and ^{13}C-NMR experiments appeared to be strongly deshielded, especially the two methine groups linked to the nitrogen atom [δ_H 4.07 (H-1 and H-5) and δ_C 72.5 (C-1), 76.6 (C-5)] and the N-CH_3 moiety [δ_H 3.42 and δ_C 48.9] (Figure 0-46). According to the literature (Lin *et al.*, 2002; Silva *et al.*, 2001; Wenkert *et al.*, 1974), the deshielding effect observed in the NMR experiments could be explained by the presence of a *N*-oxide function. Moreover, no exchangeable protons were detected in the molecule by LC-MS analysis using D_2O as eluent (see chapter 2.2.1), confirming the presence of the *N*-oxide group. The additional oxygen atom was then located on the N-CH_3 moiety and **18** was considered as a tropane alkaloid *N*-oxide.

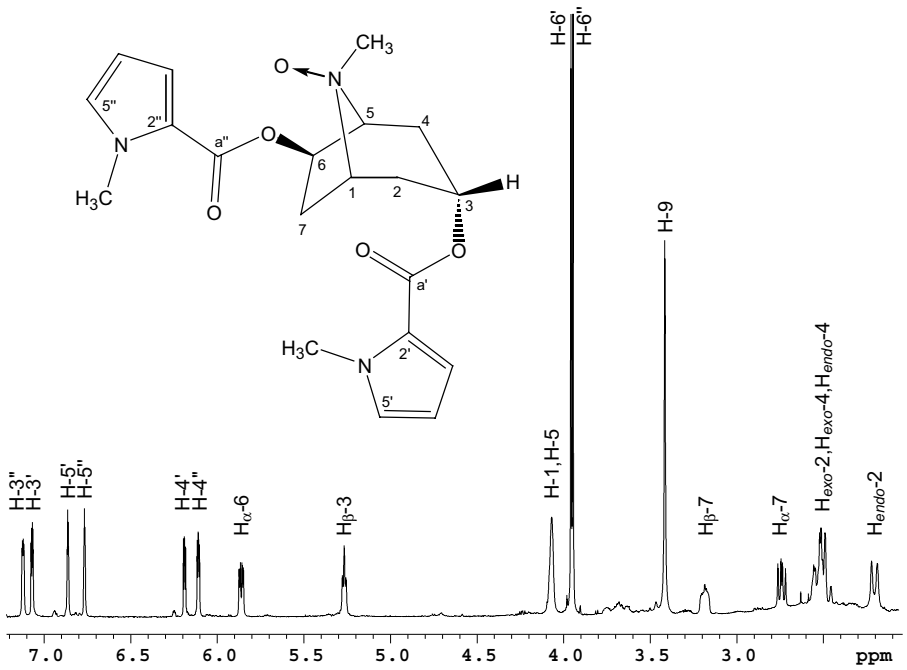


Figure 0-46

^{1}H-NMR spectrum of compound **18**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

The relative configuration was deduced by the interpretation of the recorded H-3 multiplicity and H-6 couplings, and it was confirmed by the NOESY experiment.

From the evidence of the aforementioned spectroscopic data, compound **18 (**catuabine E *N*-oxide) was identified as 3α,6β-di-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane *N*-oxide.

2.4.7. Catuabine F

Compound **9** was obtained as an amorphous solid. Its molecular formula $C_{23}H_{28}N_2O_7$ was determined by HRESMS. The EIMS gave a molecular ion at *m/z* 444 and a fragmentation pattern partially similar to that of the other isolated compounds (Figure 0-47). The appearance of a new fragment ion (*m/z* 181) suggested the presence of a novel substituent linked to the tropane nucleus.

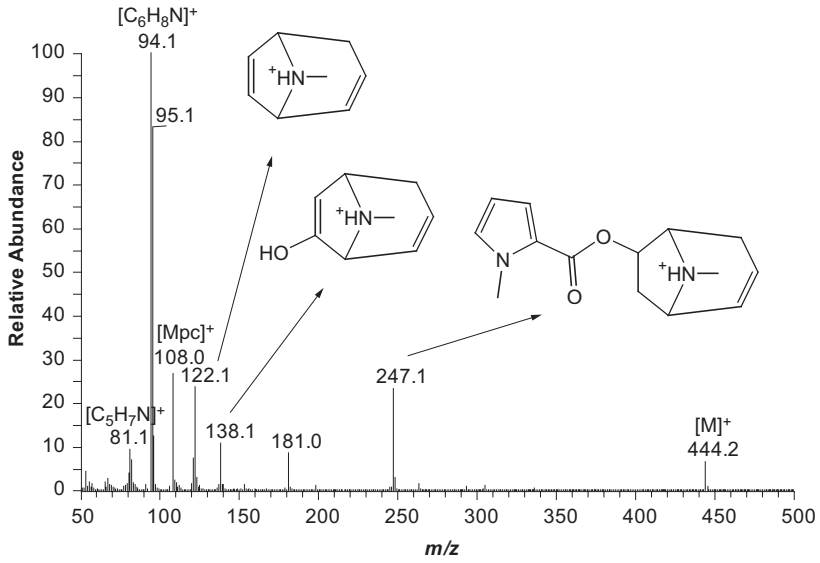


Figure 0-47

EI-MS spectrum of compound **9**. (70 eV; vaporization temperature: 50-300°C in 1 min; source temperature: 150°C; quadrupoles temperature: 70°C)

An extensive NMR analysis indicated that compound **9** was closely related to **24** since the NMR data associated to the tropane moiety and the methylpyrrole ester observed for the two compounds were superimposable (Figure 0-48; Figure 0-49). The other elements of their NMR data exhibited differences only in the nature of their substituents at the 3-position of the tropane nucleus.

gHSQC and gHMBC experiments gave evidence for a trioxygenated benzoyl moiety in the alkaloid **9**. Indeed, the remaining aromatic signal at δ_H 7.39 (2H, *s*, H-2' and H-6'), which integrated for two protons, presented long-range ^{1}H-^{13}C couplings with only three aromatic carbons at δ_C 120.7, 139.7 and 147.0 (Figure 0-50). These elements, corroborated by the molecular formula, suggested symmetry in this aromatic ring. The exact positions of the two methoxyl groups at δ_H 3.99 (6H, s, 3'-OMe and 5'-OMe) and the undetectable hydroxyl phenolic group were deduced by observation of a long-range ^{1}H–^{13}C coupling between the signal at δ_H 7.39 and an ester carbonyl at δ_C 165.6 (C-a'), showing the protons to be at 2'- and 6'-positions of the aromatic ring. Thus, this substructure was a 4-hydroxy-3,5-dimethoxybenzoyloxy moiety and its substitution at the 3-position of the tropane nucleus was confirmed by the long-range ^{1}H-^{13}C coupling between H-3 and C-a'.

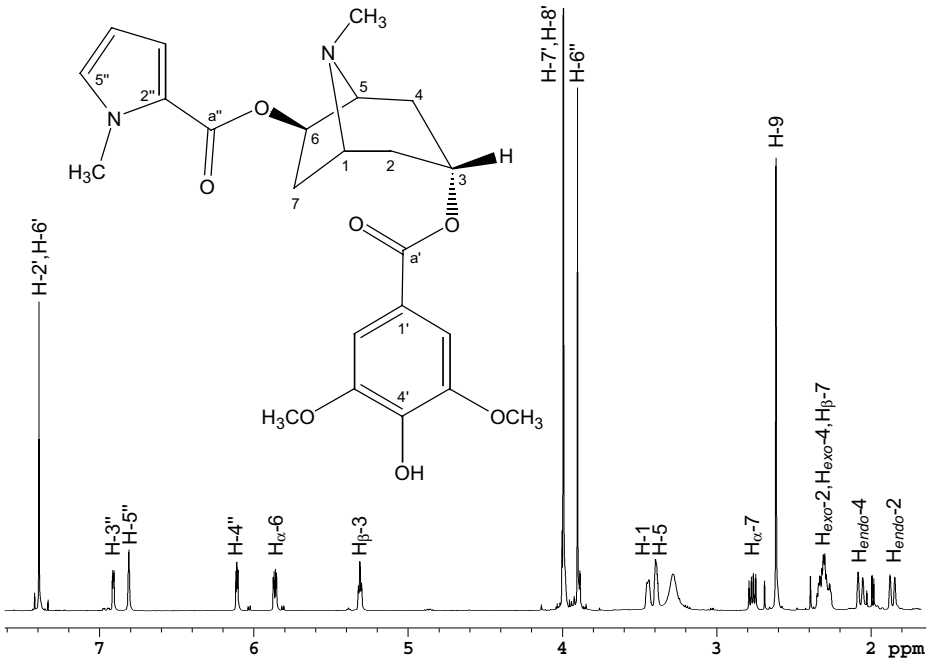


Figure 0-48

^{1}H-NMR spectrum of compound **9**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard; 1 drop of CD_3OD was previously added to facilitate solution of the sample)

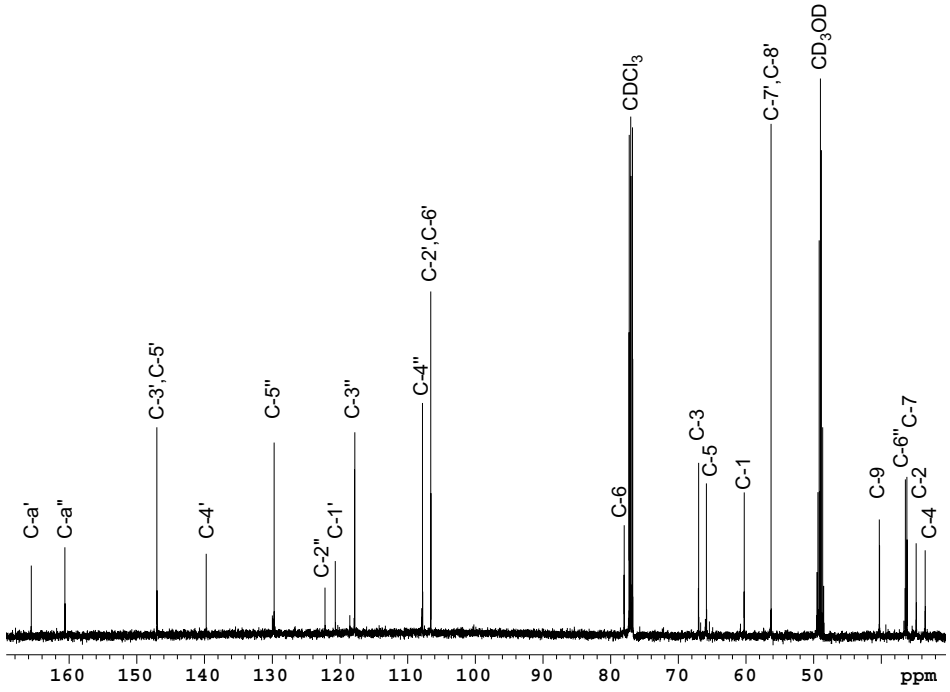


Figure 0-49

^{13}C-NMR spectrum of compound **9**. The ^{13}C assignments were ascertained by DEPT, gHSQC, and gHMBC experiments. (Spectrum recorded in $CDCl_3$ at 125.70 MHz using $CDCl_3$ as internal standard; 1 drop of CD_3OD was previously added to facilitate solution of the sample)

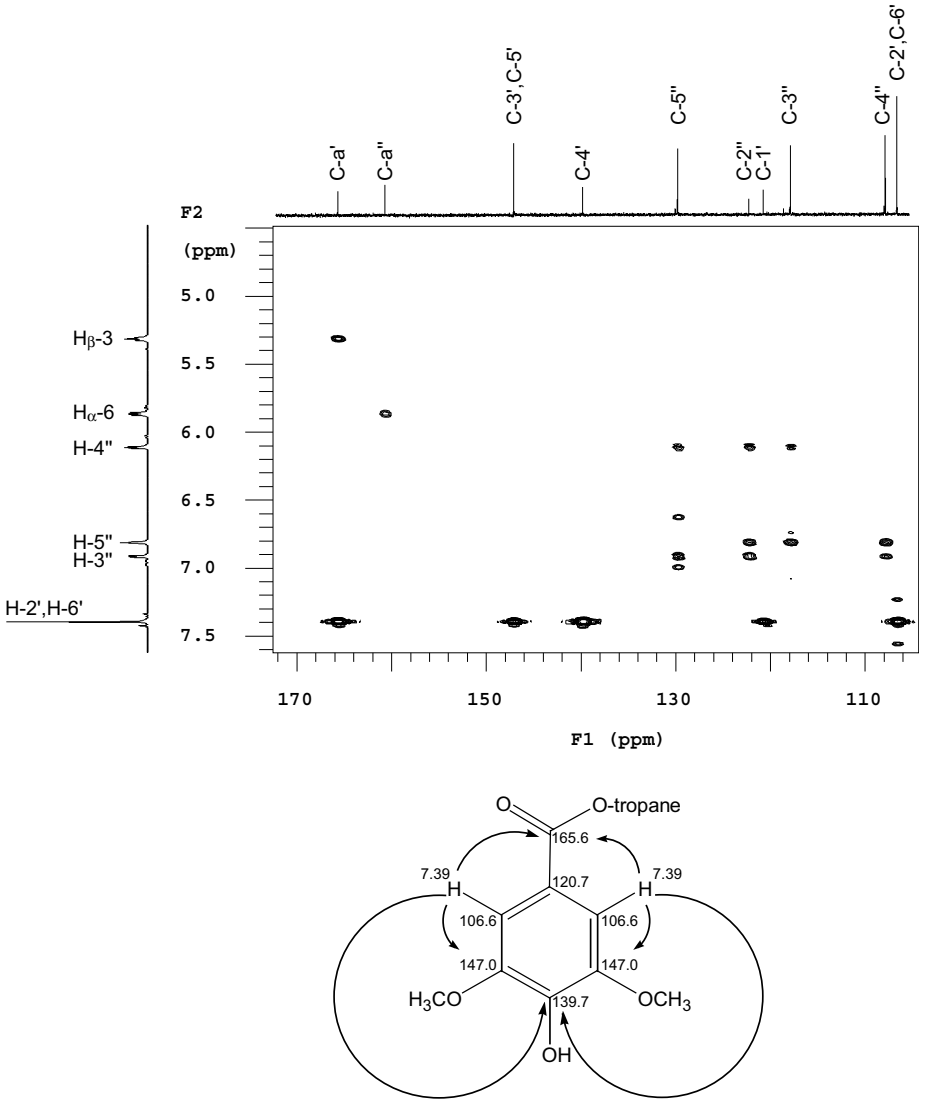


Figure 0-50

Long-range ^{1}H-^{13}C correlations of compound **9**, chemical shifts of the hydroxydimethoxybenzoic ester (δ values given in ppm) and selected gHMBC interactions.

The relative configuration of compound **6** was the same as the other isolated alkaloids. Actually, the coupling constants for H-3 (t, J = 4.9 Hz) and absence of coupling between H-6 and H-5 designated β- and α-orientations, respectively for these protons. On the basis of the above evidence, compound **9**, or catuabine F, was identified as 3α-(4-hydroxy-3,5-dimethoxybenzoyloxy)-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

2.4.8. 7β-Hydroxycatuabine F

Compound **4** was purified as an amorphous solid. HRESMS analysis of its pseudomolecular ion $[M+H]^+$ indicated the molecular formula as $C_{23}H_{29}N_2O_8$. The EIMS exhibited a molecular ion at *m/z* 460, 16 amu higher than that of **9**. The fragmentation pattern was akin to that of **4**, suggesting a similar core structure with the addition of a hydroxyl group. The analysis of NMR data demonstrated that compounds **4** and **9** were two alkaloids with the same substituents but with a different tropane core skeleton. The ^{1}H- and ^{13}C-NMR spectra of the ester substructures of these two alkaloids were superimposable, while the spectral region of their tropane centers showed diverse substitution patterns. The ^{1}H-NMR spectrum of **4** showed typical resonances for a trioxygenated tropane with signals at δ_H 5.36 (H-3β), δ_H 5.91 (H-6α) and δ_H 4.90 (H-7α) (Figure 0-51). The position of the substituents was determined by analysis of the long-range ^{1}H–^{13}C correlations as for compound **9**.

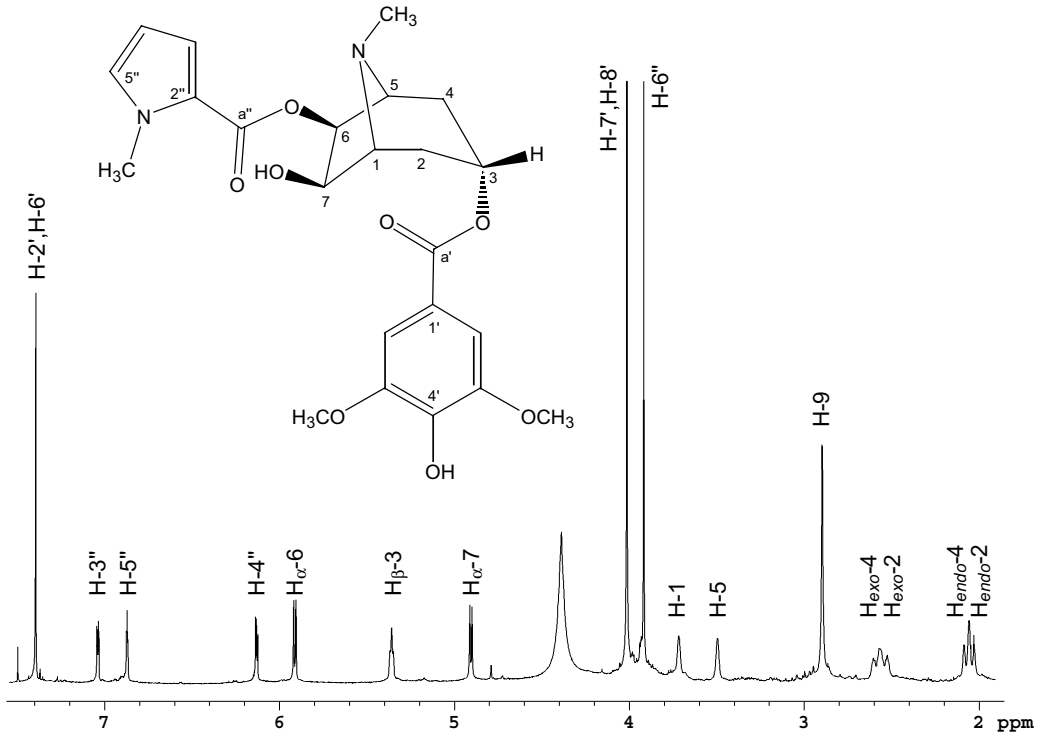


Figure 0-51

^{1}H-NMR spectrum of compound **4**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard; 1 drop of CD_3OD was previously added to facilitate solution of the sample)

The relative configuration of **4** was established by the observation of the multiplicity and the couplings of the protons at C-3, C-6 and C-7 positions, giving identical results to that of the other compounds isolated. From the evidence of the aforementioned spectroscopic data,

compound **4** (7β-hydroxycatuabine F) was identified as 3α-(4-hydroxy-3,5-dimethoxybenzoyloxy)-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-7β-hydroxytropane.

2.4.9. Catuabine G

The molecular formula of **2** was established as $C_{16}H_{22}NO_5$ by HRESMS. The NMR data of **2** differed from those of the other tropanes. In fact, only thirteen signals were observed in its ^{13}C-NMR spectrum, indicating a certain symmetry in the molecule (Figure 0-52). In addition, ^{1}H-NMR data of **2** exhibited typical resonances for a 3-hydroxyphenylacetoxy substructure (Al-Said *et al.*, 1986a; Silva *et al.*, 2001) (Figure 0-53).

Long-range ^{1}H–^{13}C couplings deduced from the gHMBC spectrum of **2** revealed links between the phenol moiety, the methylene group and the carbonyl of an ester function (δ_C 170.4) (Figure 0-54; Figure 0-55).

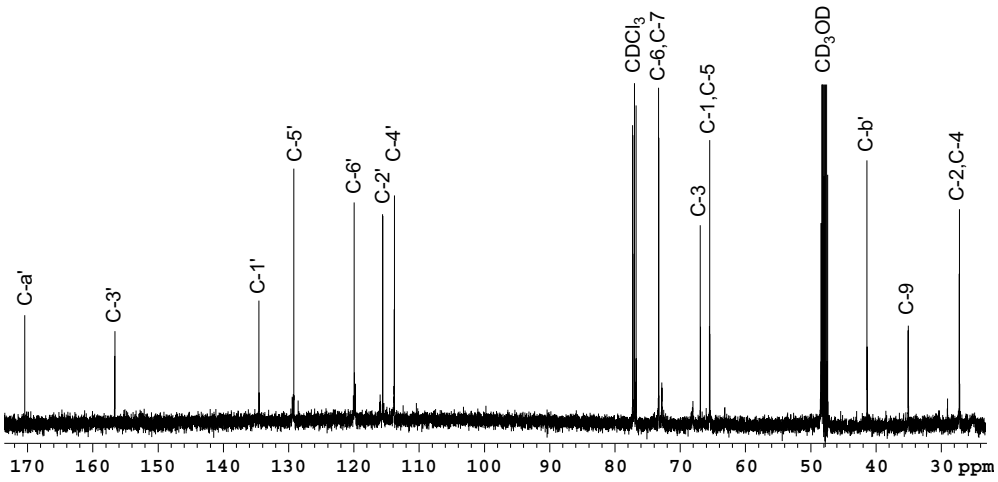


Figure 0-52

^{13}C-NMR spectrum of compound **2**. The ^{13}C assignments were ascertained by DEPT, gHSQC, and gHMBC experiments. (Spectrum recorded in $CDCl_3$ at 125.70 MHz using $CDCl_3$ as internal standard; 1 drop of CD_3OD was previously added to facilitate solution of the sample)

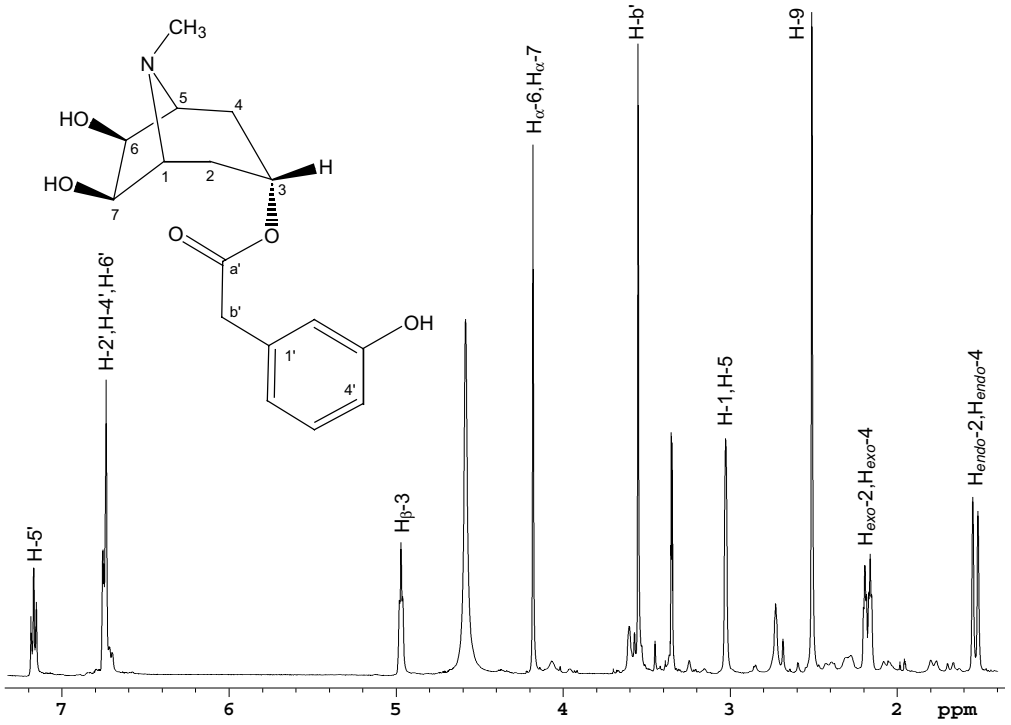


Figure 0-53

^{1}H-NMR spectrum of compound **2**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard; 1 drop of CD_3OD was previously added to facilitate solution of the sample)

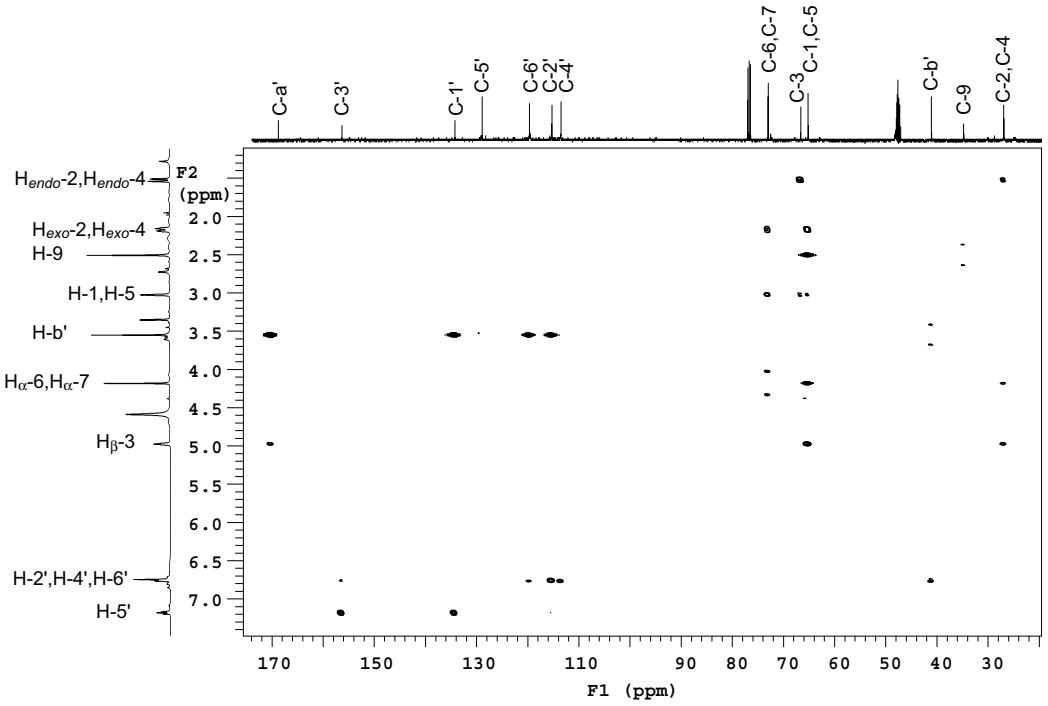


Figure 0-54

gHMBC spectrum of compound **2**.

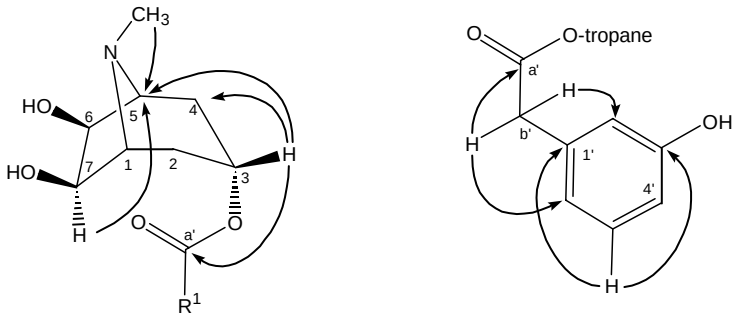


Figure 0-55

Selected long-range ^{1}H- ^{13}C correlations of compound **2**.

The presence of a 3-hydroxyphenylacetoxy substructure was further inferred from the ions observed in the EIMS at *m/z* 156 $[M - 3\text{-}OHPhCH_2CO]^+$ and 107 $[3\text{-}OHPhCH_2]^+$ (Figure 0-56).

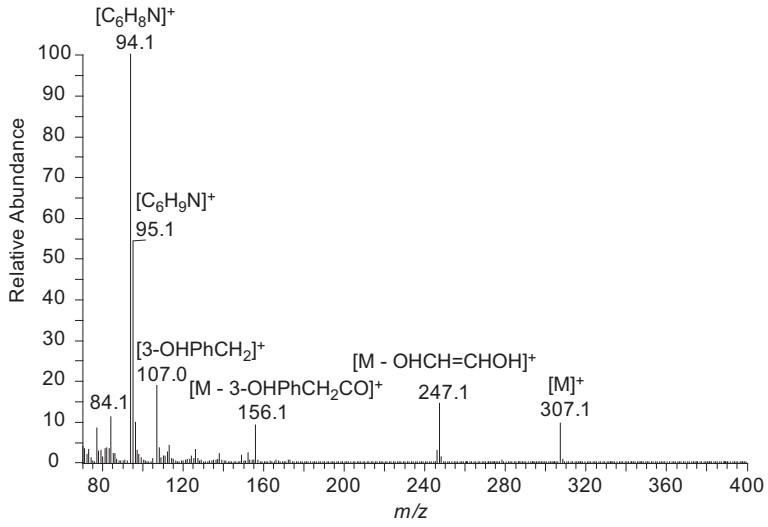


Figure 0-56

EI-MS spectrum of compound **2**. (70 eV; vaporization temperature: 50-300°C in 1 min; source temperature: 150°C; quadrupole temperature: 70°C)

Then, deduced from the reduced number of the remaining signals observed in the ^{1}H and ^{13}C-NMR spectra of **2**, the tropane substructure was characterized by the presence of a plane of symmetry. This fact was also corroborated by the integration values in the ^{1}H spectrum calculated for the tropanic signals at δ_H 1.52 (H_{endo}-2 and H_{endo}-4), 2.17 (H_{exo}-2 and H_{exo}-4), 2.51 (N-CH_3), 3.02 (H-1 and H-5), 4.18 (H-6α and H-7α) and 4.97 (H-3β). Of note was the

equivalence of the two positions C-6 and C-7, both substituted by an hydroxyl function. No coupling constant was observed between H-6 (or H-7) and the vicinal proton H-5 (or H-1), implying an β-orientation of the two hydroxyl moieties. The long-range ^{1}H-^{13}C coupling between H-3 and the ester carbonyl at δ_C 170.4 (C-a') showed attachment of the 3-hydroxyphenylacetoxy moiety to the 3-position of the tropane nucleus (Figure 0-55). Thus, the structure of alkaloid **2** was elucidated as 3α-(3-hydroxyphenylacetoxy)-6β,7β-dihydroxytropane, a new compound (catuabine G).

2.4.10. Catuabine H

Compound **13** was isolated as a white amorphous powder. High-resolution electrospray ion cyclotron resonance mass spectroscopic analysis (HRESMS) of this compound suggested a molecular formula of $C_{14}H_{20}N_2O_3$, implicating six centers of unsaturation and/or ring structures in the molecule. The ^{1}H-NMR spectrum of **13** exhibited typical resonances for a methylpyrrole substructure with signals at δ_H 3.93 (N-CH_3), δ_H 6.09 (H-4'), δ_H 6.77 (H-5') and δ_H 6.90 (H-3') (Figure 0-57).

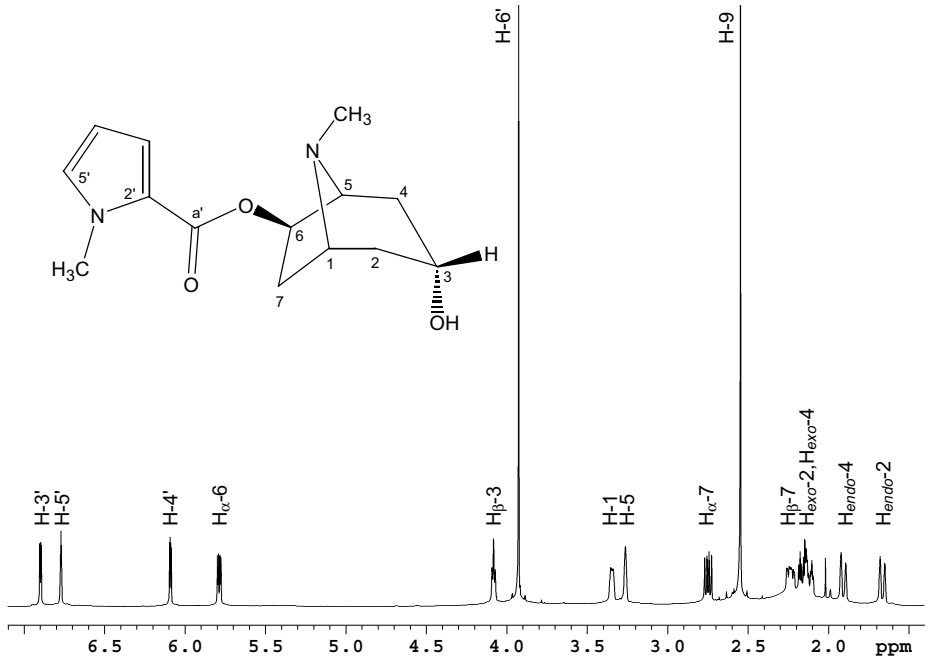


Figure 0-57

^{1}H-NMR spectrum of compound **13**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

Long-range ^{1}H-^{13}C couplings deduced from the gHMBC spectrum of **13** revealed that the pyrrolic proton at δ_H 6.90 (H-3') correlated with a carbonyl carbon at δ_C 161.2 (C-a') indicating the substitution of the methylpyrrole moiety at the 2'-position by an ester group. The UV spectrum of **13** showed a maximum of absorbance at 267.0 nm supporting the presence in the molecule of the 2-(carbonyloxy)methylpyrrole chromophore. According to the molecular formula of **13**, the remaining moiety was characterized by the presence of one nitrogen atom and two ring structures. Two methine groups and one methyl group linked to the nitrogen atom were recorded at δ_H 3.26 (H-5), 3.35 (H-1), 2.55 (N-CH_3) and δ_C 66.6 (C-5), 60.4 (C-1), 40.6 (N-CH_3), suggesting their inclusion in a tropane skeleton. The observation in the ^{1}H-NMR data of three saturated methylene moieties at δ_H 1.66, 2.12 (H-2), 1.91, 2.16 (H-4), 2.24, 2.75 (H-7) and two deshielded methine groups at δ_H 4.08 (H-3) and 5.79 (H-6) completed the characterization of the tropane core skeleton. The substitution pattern of the alkaloid substructure was deduced by extensive NMR analysis with gDQF-COSY, gHMBC and NOESY experiments. A long-range ^{1}H-^{13}C coupling associating the deshielded methine at δ_H 5.79 (H-6) and the carbon of the carbonyl function at δ_C 161.2 (C-a') implicated the location of the ester moiety at the 6-position of the tropane nucleus. The remaining oxygen atom was associated with the 3-position of the alkaloid because of its characteristic chemical shifts at δ_H 4.08 (H-3) and δ_C 64.1 (C-3). The presence of one exchangeable proton in the molecule was confirmed by its LC-MS analysis using D_2O as eluent (see chapter 2.2.1). An increase of 2 Da, due to the exchangeable hydroxyl function and the ionizing deuteron, was in fact observed in the deuterated molecule ($[M_D+D]^+$ 267 Da) when compared to the corresponding protonated molecule ($[M_H+H]^+$ 265 Da).

The stereochemical orientation of the substituents relative to the nitrogen-containing bridge, was deduced according to the multiplicity and coupling of the corresponding signals. The H-3 signal showed a triplet and coupling constant *J* of 4.9 Hz indicating the α-orientation (*i.e.*, *endo*) of the substituent at C-3.(Agar and Evans, 1976; Al-Said *et al.*, 1989b; El-Iman *et al.*, 1987) The arrangement of the substituent at C-6 was established by the analysis of the coupling constants of H-6, H-7 and H-5 protons. The H-6 proton of the disubstituted tropane alkaloid showed two couplings (*J* = 7.3, 3.4) with the two H-7 protons and it did not present any coupling with the vicinal H-5 proton. This observation implied a β-orientation (*i.e.*, *exo*) of the substituent and a dihedral angle close to 90° between H-5 and H-6α. The relative stereochemistry of the tropane substructure was also corroborated by analysis of its NOESY spectrum.

Thus, the structure of catuabine H (**13**) is 3α-hydroxy-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

2.4.11. Isocatuabine H

Compound **11** was purified as a white amorphous powder and was assigned the same molecular formula as **13**, $C_{14}H_{20}N_2O_3$. The spectroscopic data of **11**, close to these reported for **13** (Figure 0-58), were consistent with a general structure containing a central tropane moiety dioxygenated at C-3 and C-6, and esterified by one methylpyrrole acid in the 6-position.

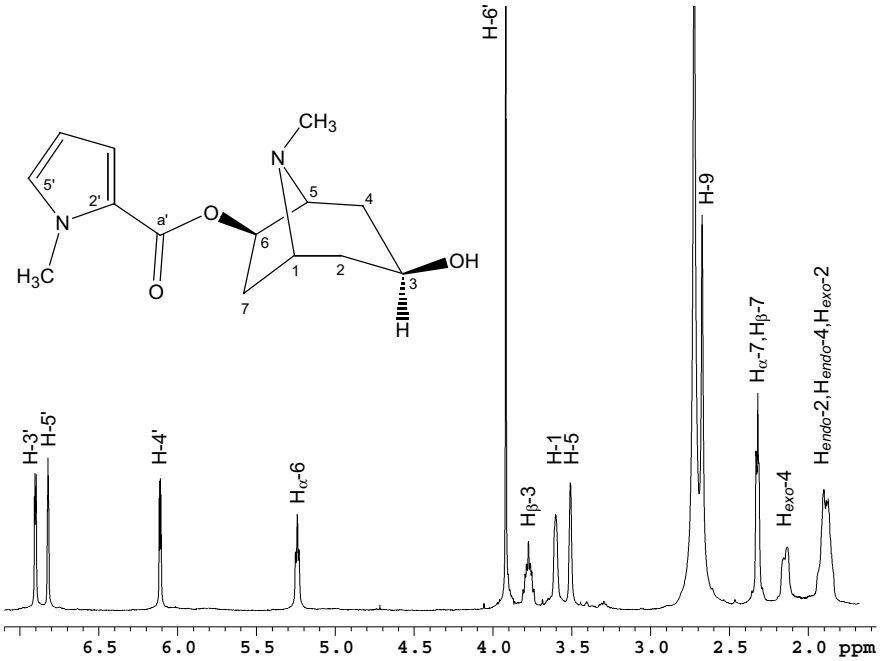


Figure 0-58

^{1}H-NMR spectrum of compound **11**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

The main difference between the NMR data of compounds **13** and **11** concerned the multiplicity (triplet and doubled triplet, respectively) and the coupling constants (J = 4.9 Hz and J = 10.8 and 5.9 Hz, respectively) recorded for their H-3 proton signals. The modification of the multiplicity of the H-3 proton signal according to its relative configuration is well-known in the literature of the tropane alkaloid series (Agar and Evans, 1976; Al-Said *et al.*, 1986a; Alyahya *et al.*, 1979). As reported for **13**, the α-orientation of the hydroxyl group in C-3 was associated with a triplet multiplicity, while the doubled-triplet multiplicity of H-3 for compound **11** was related to the β-orientation of the substituent (Figure 0-59). The presence of NOE interactions between the protons H-6 and H-3 confirmed that these groups were located on the same side of the molecule (α-position).

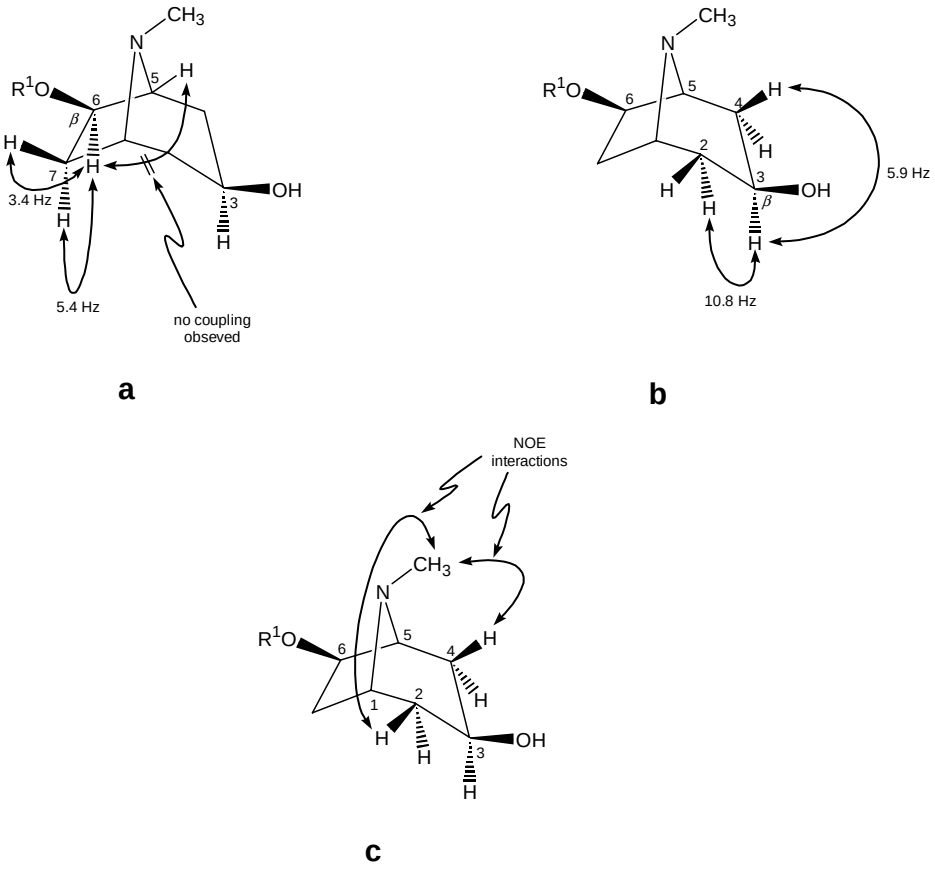


Figure 0-59

Relative configuration of compound **11**, as deduced from ^{1}H-NMR coupling constants (**a** and **b**) as well as NOESY interactions (**c**).

Hence, the structure of **11** (isocatuabine H) was deduced as 3β-hydroxy-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

2.4.12. 7β-Hydroxycatuabine H

Alkaloid **7** was purified as a white amorphous powder and also exhibited spectroscopic data similar to those of catuabine H (**13**). The HRES mass spectrum of this compound showed a protonated molecular ion at *m/z* 281.1492, 15.9946 amu higher than that of **13**, suggesting the occurrence of an additional oxygen atom in the molecule ($C_{14}H_{20}N_2O_4$). The NMR data of these two compounds were also closely related, indicating the presence in **7** of a central tropane moiety esterified by one methylpyrrole subunit. Except for proton H-7, the ^{1}H-NMR spectrum of the tropane nucleus of **7** exhibited similar chemical shifts to **13** (Figure 0-60). Actually, this spectrum showed typical resonances for a tropane alkaloid skeleton trisubstituted at C-3, C-6 and also C-7 positions with signals at δ_H 4.11 (H-3β), δ_H 5.75 (H-

6α) and δ_H 4.84 (H-7α), respectively. According to the molecular formula and the values of the chemical shifts associated with the 7-position (δ_H 4.84 and δ_C 75.8), a hydroxyl substituent was required at this location.

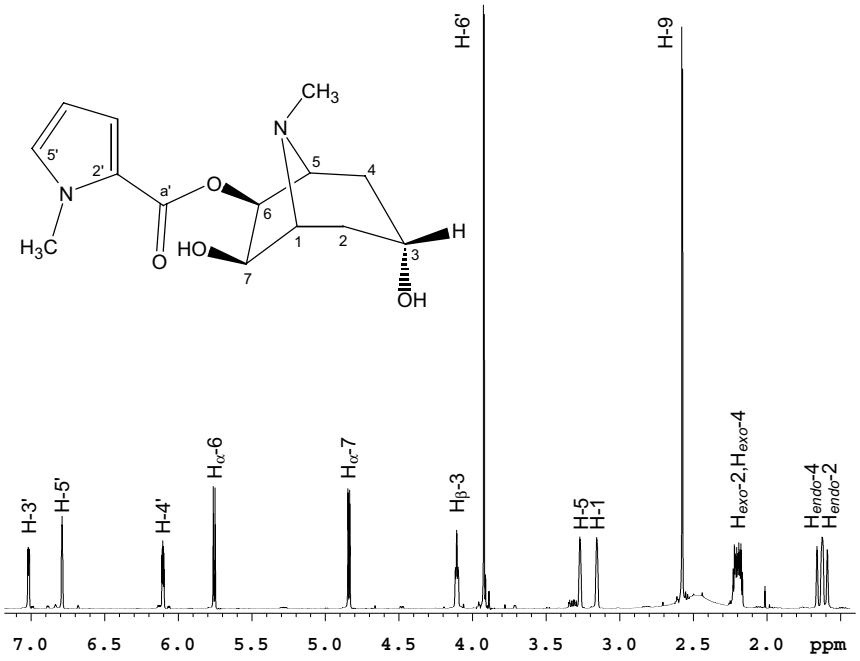


Figure 0-60

^{1}H-NMR spectrum of compound **7**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

The α-orientation of the hydroxyl group in C-3 was resolved from the multiplicity (triplet) and the coupling constant (J = 4.9 Hz) of the H-3 proton signal. The α-stereochemistry of protons at C-6 and C-7 position was deduced by the lack of any coupling constant with vicinal protons H-5 and H-1, respectively, and confirmed by analysis of the NOESY spectrum.

Hence, the structure of **7** (7β-hydroxycatuabine H) was deduced as 3α,7β-dihydroxy-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

2.4.13. 7α-Hydroxycatuabine H

Compound **8**, purified as a white amorphous solid, had the same molecular formula as **7** ($C_{14}H_{20}N_2O_4$) as deduced from HRESMS analysis. The NMR data strongly resembled to those of **7**, consistent with a general structure containing a central tropane moiety trioxygenated at C-3, C-6 and C-7, and esterified by a methylpyrrolic acid in the 6-position (Figure 0-61).

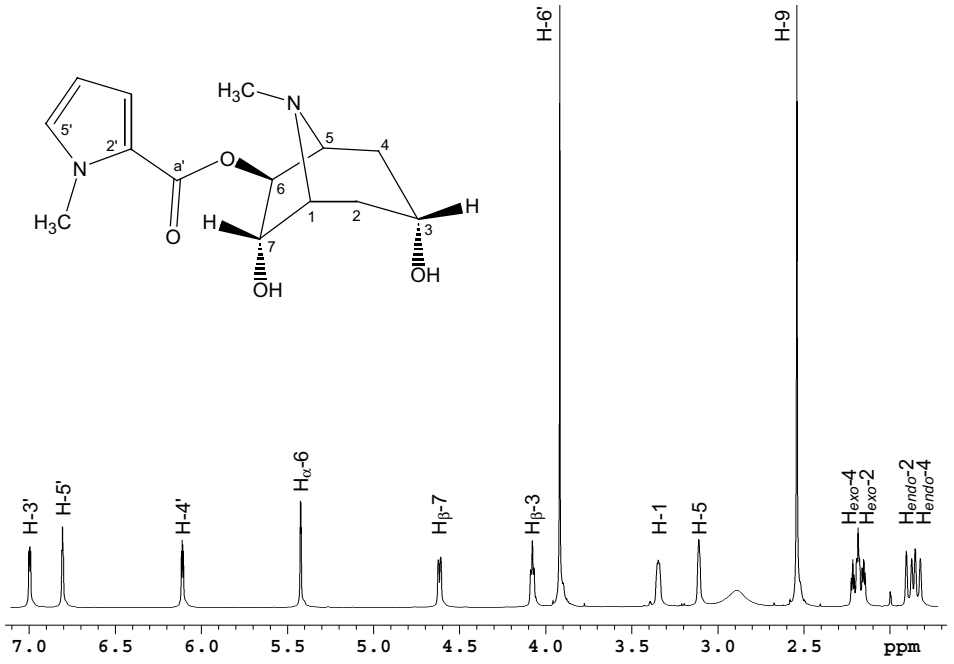


Figure 0-61

^{1}H-NMR spectrum of compound **8**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

NMR analysis of the two compounds revealed only slight differences, essentially concerning the multiplicity and the couplings of some proton signals of the tropane core skeleton. The absence of any coupling constant between H-6 and H-7 in compound **8** suggested a vicinal angular relationship around 90° for the two protons. In addition, unlike the previous compounds, a coupling constant was observed between H-7 and H-1 (J = 6.4 Hz) indicating a dihedral angle close to 0° between the two protons (Figure 0-62). The presence of NOE interactions between the protons N-CH_3 and H-7 confirmed the β-orientation of this latter. In addition, this NOE contact suggested that compound **8** distinguished itself from the other elucidated compounds by the orientation of the tropanic methyl group.

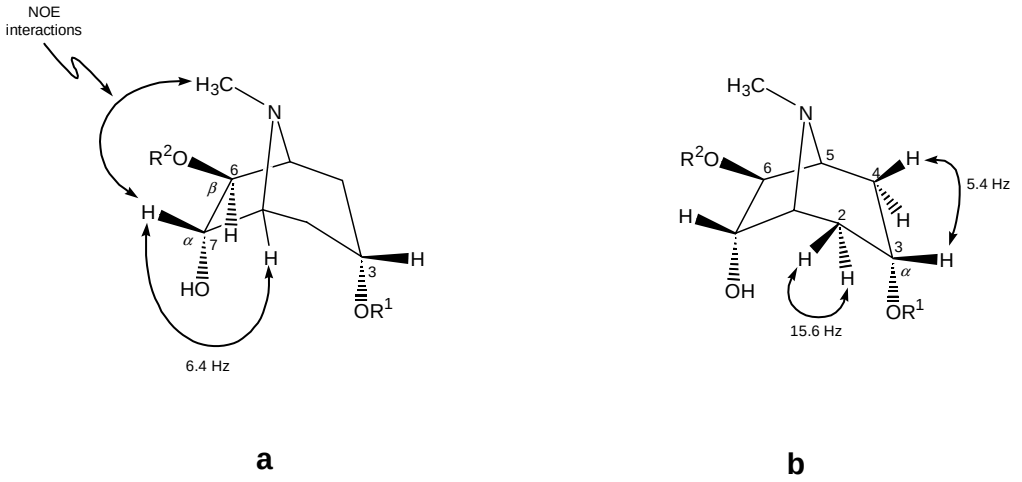


Figure 0-62

Relative configuration of compound **8**, as deduced from ^{1}H-NMR coupling constants (**a** and **b**) as well as NOESY interactions (**a**).

On the basis of the above evidence, compound **8**, or 7α-hydroxycatuabine H, was identified as 3α,7α-dihydroxy-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

2.4.14. Catuabine I

Compound **12** was isolated as a white amorphous powder. According to a HRESMS experiment, it gave a molecular formula of $C_{14}H_{20}N_2O_3$ (Δ -0.1 mamu), corresponding to that observed for compounds **13** and **11**. The NMR spectroscopic data of **12** were consistent with a C-3 and C-6 dioxygenated tropane alkaloid substituted by a methylpyrrole ester. Nevertheless, in this molecule, the signal at δ_H 5.15, typically shifted downfield with respect to nonesterified compounds, like **13** and **11**, indicated esterification at C-3 (δ_C 65.3) rather than a free alcohol (Al-Said *et al.*, 1986b; El-Iman *et al.*, 1987; Payo-Hill *et al.*, 2000) (Figure 0-63). Conversely, the proton at 6-position exhibited a resonance at δ_H 4.72, indicating a hydroxyl group at C-6 (δ_C 74.8). A long-range ^{1}H-^{13}C correlation between the oxygenated methine at δ_H 5.15 (H-3) and the carbonyl signal at δ_C 160.2 (C-a') confirmed that the pyrrole unit substituted the tropane moiety in the C-3 position.

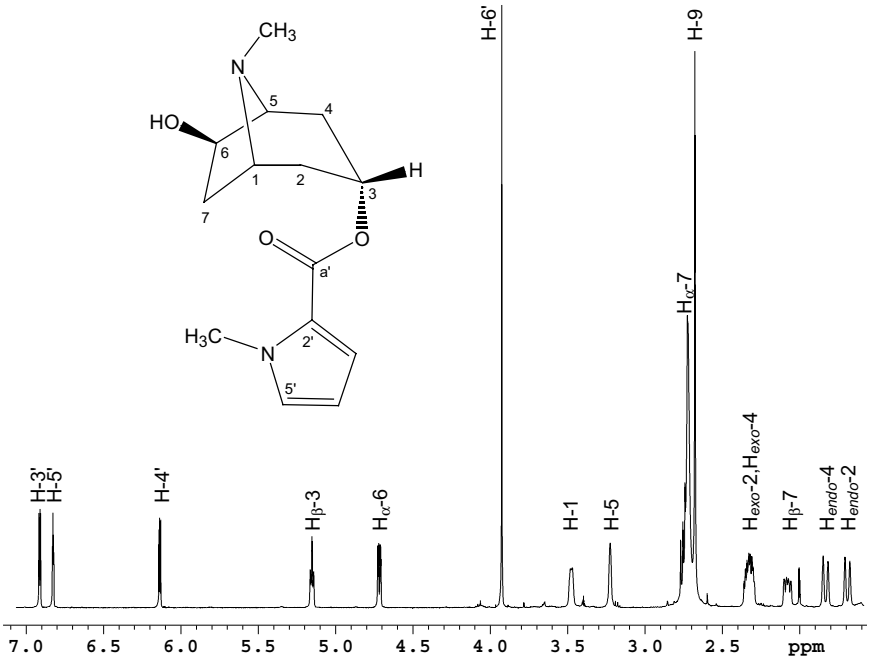


Figure 0-63

^{1}H-NMR spectrum of compound **12**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

By analysis of its NOESY spectrum, the stereochemistry of **12** was assigned as in **13**. The relative configuration was corroborated according to the multiplicity of H-3 and coupling between H-6 and H-5.

Thus, the structure of catuabine I (**12**) is 6β-hydroxy-3α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

2.4.15. 7β-Hydroxycatuabine I

The molecular formula of compound **6** was established as $C_{14}H_{20}N_2O_4$ by HRESMS suggesting the occurrence of an additional oxygen atom in the alkaloid with respect to compound **12**. The observation of only eleven signals in ^{13}C-NMR spectrum of **6** indicated a symmetrical part in the molecule (Figure 0-64). This suggested the equivalence of the C-6 and C-7 positions and, according to the NMR data, the additional oxygen was located at the 7-position (Figure 0-65). Thus, the tropane moiety was substituted by two hydroxyl functions in positions C-6 and C-7.

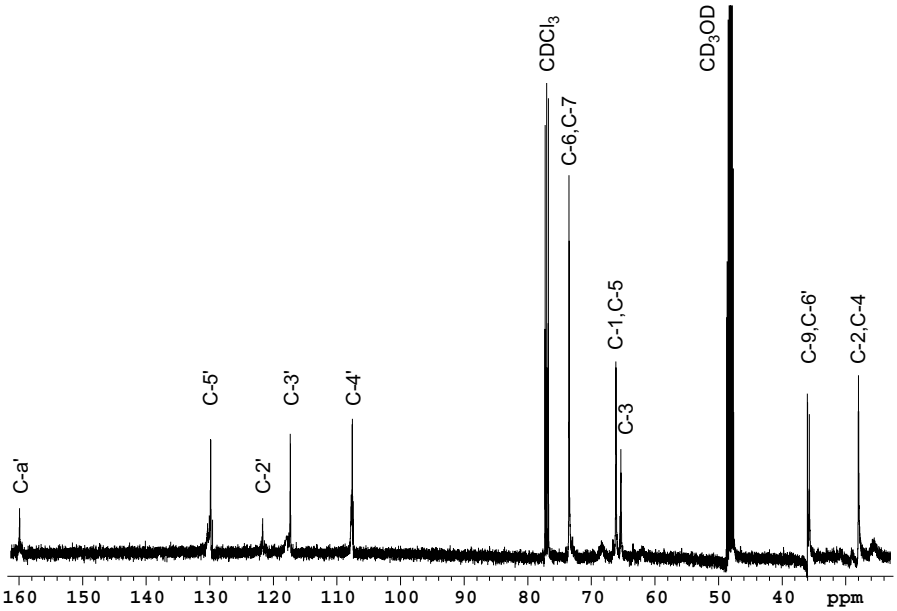


Figure 0-64

^{13}C-NMR spectrum of compound **6**. The ^{13}C assignments were ascertained by DEPT, gHSQC, and gHMBC experiments. (Spectrum recorded in $CDCl_3$ at 125.70 MHz using $CDCl_3$ as internal standard; 1 drop of CD_3OD was previously added to facilitate solution of the sample)

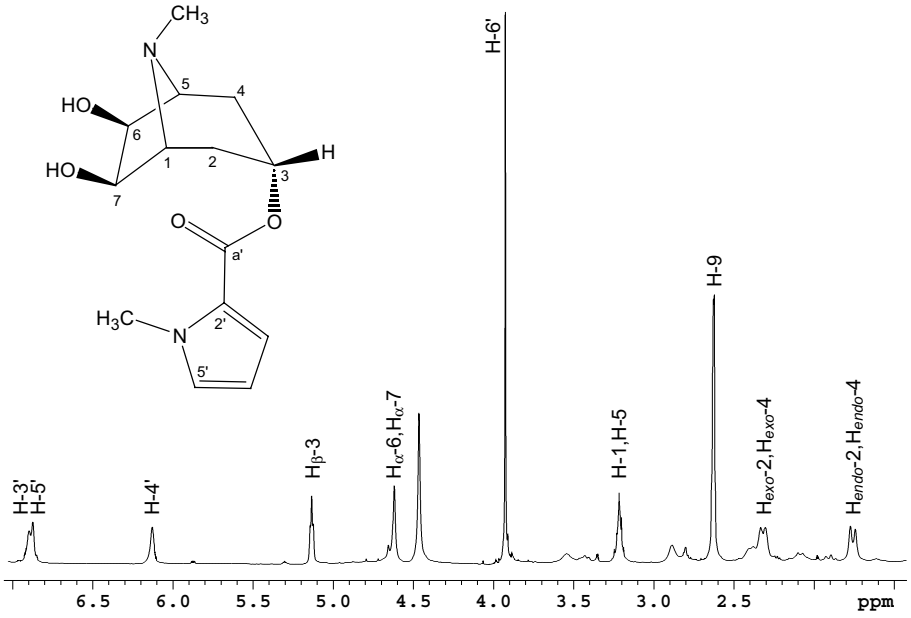


Figure 0-65

^{1}H-NMR spectrum of compound **6**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard; 1 drop of CD_3OD was previously added to facilitate solution of the sample)

No coupling constant was observed between H-6 (or H-7) and the vicinal proton H-5 (or H-1), implicating a β-orientation of the two hydroxyl groups. As observed for compound **12**, the gHMBC data of **6** were consistent with the substitution of the tropane moiety at the C-3 position by a methylpyrrole ester.

The structure of alkaloid **6** was elucidated as 6β,7β-dihydroxy-3α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane, a new compound named 7β-hydroxycatuabine I.

2.4.16. Vaccinine A

HRESMS experiments of alkaloid **15** gave $C_{14}H_{20}N_2O_3$ as molecular formula. This compound exhibited spectroscopic data similar to those of the other isolated tropane alkaloids (Figure 0-66; Figure 0-67).

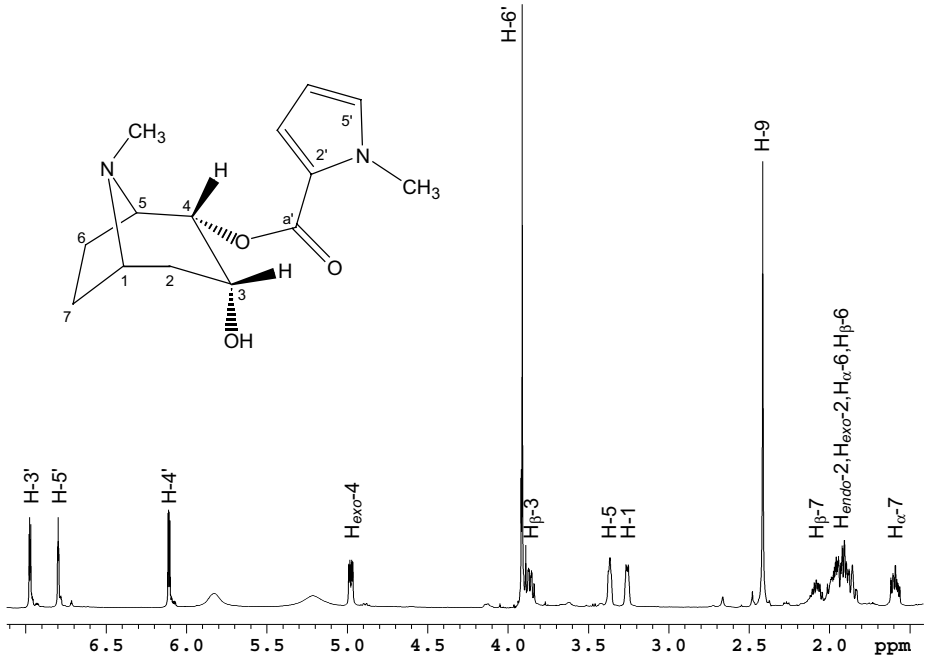


Figure 0-66

^{1}H-NMR spectrum of compound **15**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

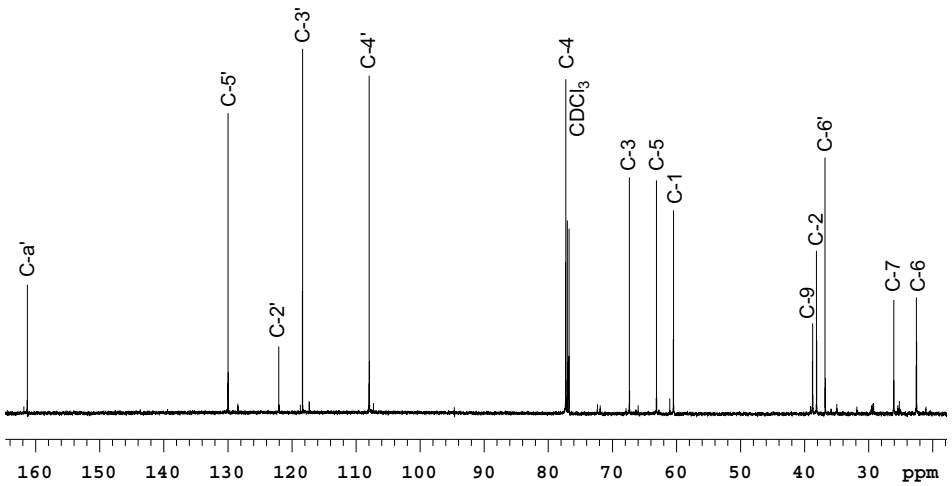


Figure 0-67

^{13}C-NMR spectrum of compound **15**. The ^{13}C assignments were ascertained by DEPT, gHSQC, and gHMBC experiments. (Spectrum recorded in $CDCl_3$ at 125.70 MHz using $CDCl_3$ as internal standard)

As previously reported for compound **13**, analysis of the gHMBC spectrum of **15** led to the identification of the two methine groups at δ_H 3.26 (H-1) and 3.37 (H-5). Their linkage to the nitrogen atom were confirmed by the observation of long-range ^{1}H-^{13}C correlations between the protons at δ_H 2.54 (N-CH_3) and the C-1 (δ_C 60.5) and C-5 (63.1). Nevertheless, in contrast to the other elucidated alkaloids, the gDQF-COSY spectrum of **15** showed a partial spin system implicating two oxygenated methine moieties, at δ_H 4.98 and 3.87, and one saturated methylene group at δ_H 1.86 and 1.96 (Figure 0-68). This observation suggested that these three latter structural elements were located on the 6-center ring of the tropane skeleton.

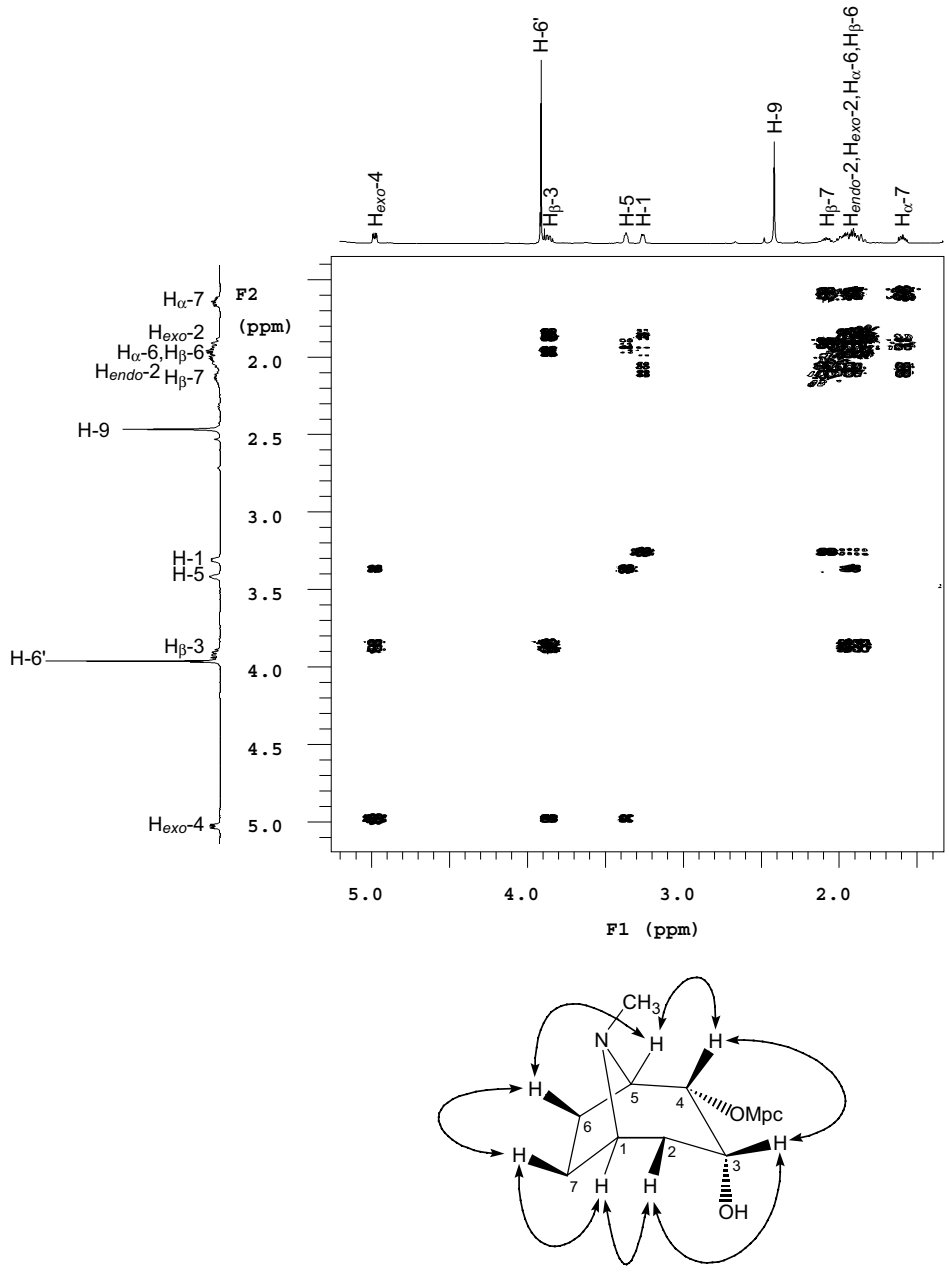


Figure 0-68

gDQF-COSY spectrum of compound **15** showing a single spin system.

Analysis of the remaining NMR data corroborated the presence of two saturated methylene groups at 6- and 7-positions of the tropane core skeleton. A long-range ^{1}H-^{13}C correlation between the oxygenated methine at δ_H 4.98 (H-4) and the carbonyl signal at δ_C 161.3 (C-a') indicated that the methylpyrrolic moiety substituted the C-4 position of the tropane. The signal at δ_H 4.98, typically shifted downfield, indicated an esterification at C-4 (δ_C 77.3) rather than a hydroxyl group.

The relative configuration of **15** was deduced by a NOESY experiment and the recorded coupling constants (Figure 0-69). Concerning the protons H-3 and H-4, their coupling constant was measured as 8.8 Hz, supporting a dihedral angle close to 0° or 180° between the two methine groups. The observation of a spatial contact between H-3 and H-4 removed the 180° dihedral angle possibility. In 1971, Johns and co-workers reported the isolation of a new nortropane alkaloid substitued at C-2 and C-3 from the leaves of *Peripentadenia mearsii* (Euphorbiaceae) (Johns *et al.*, 1971). They considered the 9.0 Hz coupling between H-2 and H-3 as a *trans* diaxial arrangement of the two protons and elucidated the structure as 2α-benzoyloxy-3β-hydroxynortropane.

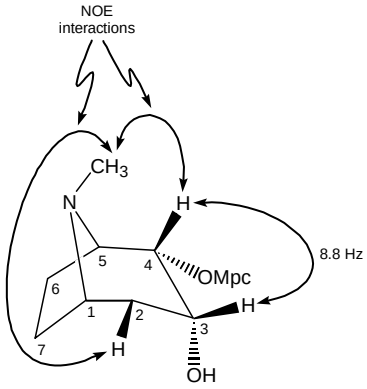


Figure 0-69

Relative configuration of compound **15**, as deduced from ^{1}H-NMR coupling constants and NOESY interactions.

On the basis of the above evidence, the structure of **15** (vaccinine A) was elucidated as 3α-hydroxy-4α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

2.4.17. Vaccinine B

Compound **14** had the same molecular formula as **11**, **12**, **13** and **15** ($C_{14}H_{20}N_2O_3$) as deduced from HRESMS analysis. Its spectroscopic data were consistent with a dioxygenated tropanic structure substituted by a methylpyrrole ester (Figure 0-70).

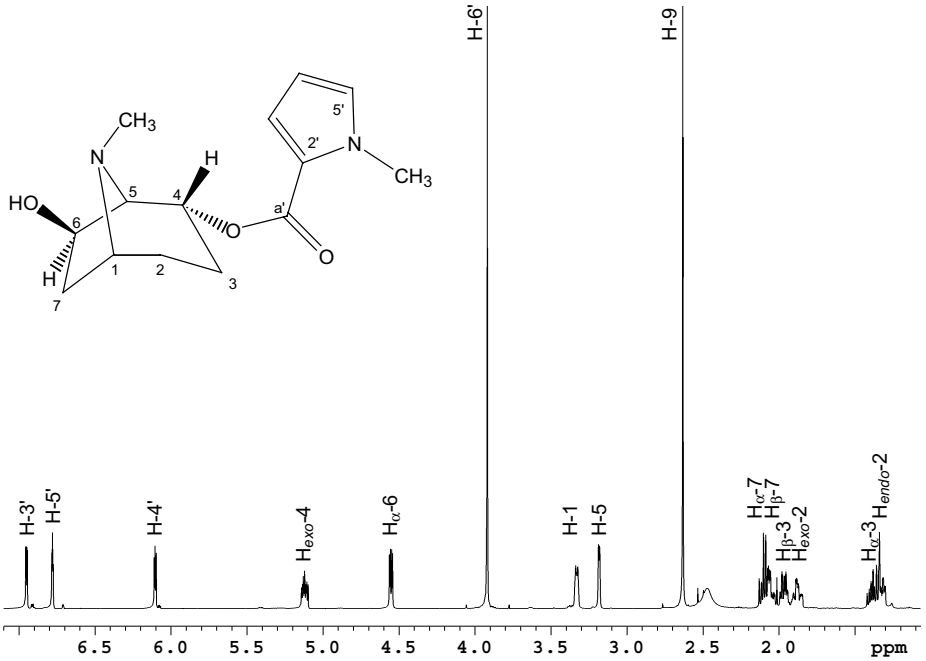


Figure 0-70

^{1}H-NMR spectrum of compound **14**. (Spectrum recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard)

The gDQF-COSY spectrum of **14** suggested that the substituents were located on the same side as the *N*-methine group at δ_H 3.18 (H-5) implicating oxygenation of the 4 and 6 positions of the tropane skeleton (Figure 0-71). The methylpyrrole ester position was deduced by the long-range ^{1}H-^{13}C correlation between H-4 (δ_H 5.12) and C-a' (δ_C 160.4), and the downfield shift of the H-4 signal. The presence of NOE interactions between the N-CH_3 protons and H-4 indicated that these groups were located on the same side of the molecule (β-orientation of H-4).

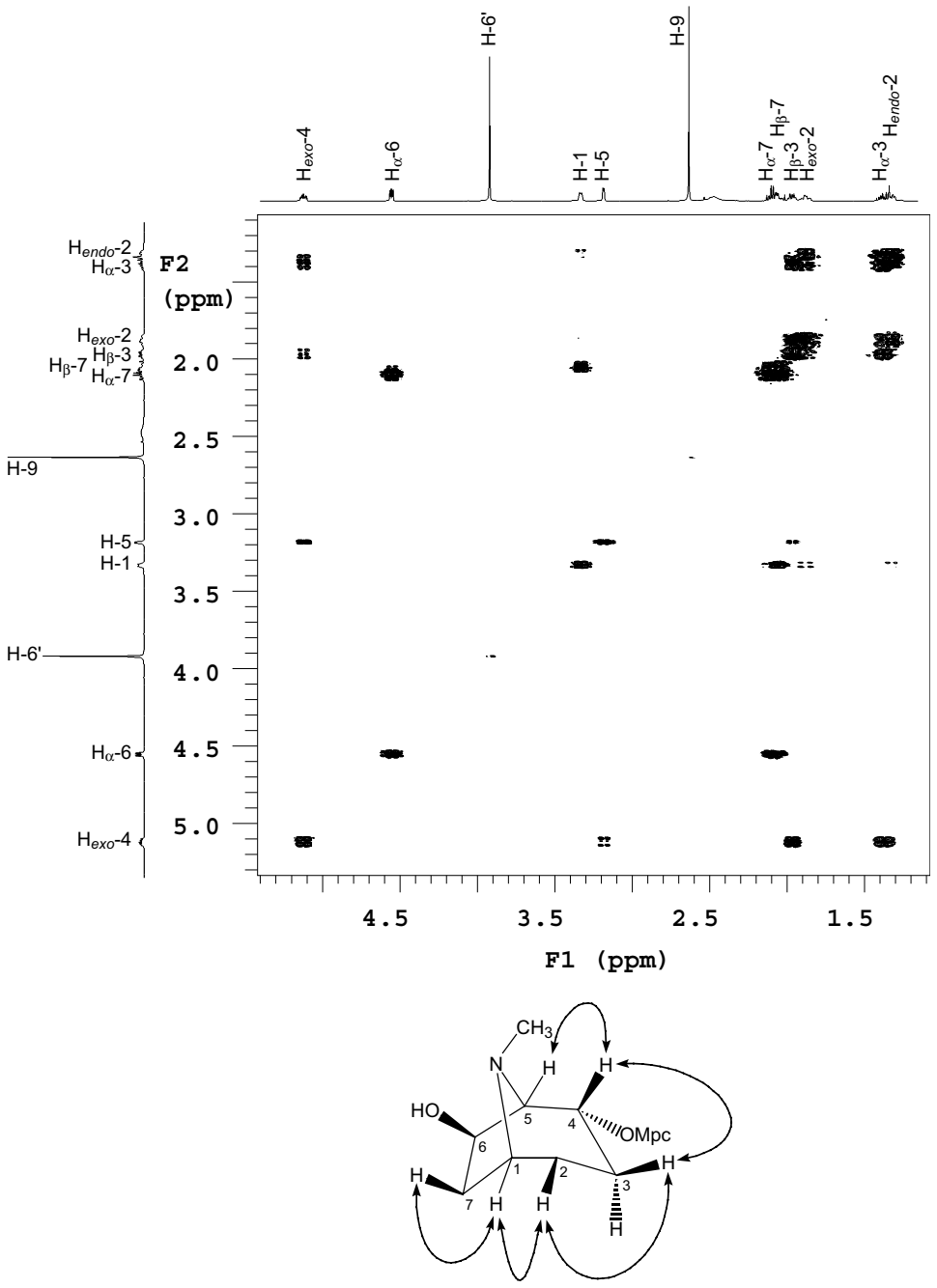


Figure 0-71

gDQF-COSY spectrum of compound **14** showing a single spin system.

The relative configuration of H-6 was elucidated according to the absence of coupling constants with H-5 and to the NOESY experiment.

Hence, the structure of **14** (vaccinine B) was deduced as 6α-hydroxy-4α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane.

2.5. Biological and biochemical activities

2.5.1. TLC bioautographic assays

The different crude extracts of the bark of *E.vacciniifolium* were submitted to four TLC bioautographic assays to evaluate their potential bioactivities. These assays are reported in the experimental part (VI.6) and they give an evaluation of the following activities: antifungal activities against *Cladosporium cucumerinum* and *Candida albicans*; antibacterial activity against *Bacillus subtilis*; acetylcholinesterase (AChE) inhibitory activity. The results of the bioassays are reported in Table 0-8.

Table 0-8

Antifungal, antibacterial and AChE inhibitory activities of extracts from the bark of *E. vacciniifolium*.

extracts	*Cladosporium cucumerinum*[d]	*Candida albicans*[d]	*Bacillus subtilis*[d]	AChE[d]
Alk. $CHCl_3$	-	-	-	(+)
DCM	-	-	-	-
MeOH	-	-	-	(+)
Water[a]	-	-	-	(+)
Water[b]	-	-	-	(+)
MeOH[c]	-	-	-	-
Water[a, c]	-	-	-	-
Water[b, c]	-	-	-	-

[a] maceration performed during 24 hs at room temperature.
[b] infusion performed during 1 hr at 100°C.
[c] Tannin-free extracts.
[d] Tested amount 100 μg of crude extract. (-, inactive; (+), weak activity; +, medium activity; ++, strong activity)

At a concentration of 100 μg on a silica gel TLC plate, no extracts showed either antifungal or antibacterial activity.

The enriched alkaloid extract exhibited weak AChE inhibitory activity. This marginal bioactivity appeared as white spots on a purple background (TLC). Spots with higher R_f

(0.45-0.75) seemed to be more active than the other spots with lower R_f (0.1-0.40), since the responses were more intense. This variation between apolar and polar compounds could be associated with their intrinsic activity or with their different concentrations in the extract.

The dichloromethane extract showed no AChE inhibitory activity, while the methanol extract and the two aqueous extracts demonstrated a moderate inhibition of the enzyme. This activity was only exhibited by very polar substances (R_f : 0.05-0.25), suggesting the potential presence of polyphenolic compounds which could primarily react with the proteins of the enzyme. The reactivity of polyphenolic compounds such as tannins toward proteins is well known and several papers have reported the interference of these molecules with bioassays (Wall *et al.*, 1996). The presence of tannins in the methanolic and aqueous extracts was detected on TLC by using various reagents. Under UV-light, galloyl esters and gallotannins appeared as violet fluorescent spots that are enhanced on fumigation with ammonia vapour. Ellagic acid produced a violet spot that darkens on exposure to ammonia vapour. The $FeCl_3/K_3Fe(CN)_6$-spray reagent was also used to detect tannins. This reagent is not a specific reagent and gives positive results for other phenolic compounds as blue spots. Another polyvalent reagent (Godin's reagent) gave red/pink spots with flavan-3-ols and condensed tannins. After the tannin detection, their removal was undertaken before retesting the extracts with AChE. Polyamide column chromatography, Sephadex LH-20, polyvinylpyrrolidone (PVP), or collagen have been used by various workers to remove tannins from plant extracts (Wall *et al.*, 1996). In the present study, the collagen (hide powder) method was employed (see chapter VI.5.3). After effective tannin removal, the AChE inhibitory activity of the methanolic and aqueous extracts disappeared. Thus, the activity of these extracts was primarily due to the reactivity of these polyphenolic compounds with the proteins of the enzyme.

Isolated compounds **2**, **4**, **6**-**9**, **11**-**15**, **18**, **20**-**24** were tested as inhibitors of AChE, since the alkaloid extract showed a certain activity against the enzyme. The inhibitions were evaluated by observing the activity of geometrical dilutions of the pure compounds .

Table 0-9

Semi-quantitative estimation of the AChE inhibitory activity of the isolated compounds from *E. vacciniifolium*.

Compound	10 μg[a]	1 μg[a]	0.1 μg[a]	0.01 μg[a]
2	-	-	-	-
4	-	-	-	-
6	-	-	-	-
7	-	-	-	-
8	-	-	-	-
9	-	-	-	-
11	-	-	-	-
12	-	-	-	-
13	-	-	-	-
14	(+)	-	-	-
15	-	-	-	-
18	-	-	-	-
20	(+)	-	-	-
21	(+)	-	-	-
22	(+)	-	-	-
23	(+)	-	-	-
24	+	(+)	-	-
Galanthamine[b]	++	++	++	++

[a] Amounts spotted in μg of pure compounds. (-, inactive; (+), weak activity; +, medium activity; ++, strong activity)

[b] Positive control.

Compounds **14** and **20**-**23** were equally active against AChE down to 10 μg but weaker than the reference compound **24**. The activity of this latter was clearly noticeable down to 1 μg, even though this value was much lower than the positive standard galanthamine (active down to 0.01 μg). To our knowledge, this inhibition property has not been reported previously for any tropane alkaloids.

2.5.2. Inhibitory activity against Phosphodiesterase (PDE)

With the aim of finding out new compounds for the treatment of erectile dysfunction, the crude extracts of *E. vacciniifolium* were evaluated for their specific inhibitory activity against PDE2 and PDE5. The assays procedures are reported in the experimental part (chapter 6.2) and the experimental results of these primary tests are illustrated in Table 0-10.

Table 0-10

Experimental results of primary biochemical assays on the inhibition of activity of phosphodiesterases.

extracts	PDE2[a]	PDE5[a]
Alk. $CHCl_3$	28%	11%
DCM	18%	19%
MeOH	12%	6%
MeOH[b]	28%	28%

[a] % of enzyme inhibition at a sample concentration of 100 μM. (Significant criteria: ≥50% of maximal inhibition)
[b] Tannin-free extract.

All extracts showed a weak inhibitory activity against PDE2 and PDE5 but none of the results met significance criteria at concentrations used. Alkaloid and methanolic extracts seemed to be more active against PDE2 than PDE5 but these inhibition differences were not sufficient to provide a selectivity between the two isoenzymes. Semi-quantitative data (*e.g.*, estimated IC50, Ki and nH) were not applicable (concentration range of 4 log units) and secondary functional assays were not carried out because inhibitory activity in primary assays was <50% at 1 log unit below initial test concentration (100 μM). Since the results of these primary biochemical assays were negative and the cost of testing was high, the tests on isolated compounds were not carried out in this study.

2.5.3. Nitric oxide (NO) induction

The potential regulation of nitric oxide synthase (NOS) expression by different extracts and isolated compounds of *E. vacciniifolium* was evaluated. This expression was calculated as the improvement of the activity of a NOS III gene promoter fragment transiently and stably transfected into EA.hy 926 cells. The detailed test methodologies are described in chapter VI.6.3.

Transfected cells were incubated with or without extracts and pure compounds for 12 hs, and cytoplasmatic cell extracts were prepared. The relative luciferase activity (corrected with renilla-luciferase activity) was taken as a measure of NOS III promoter activity. Figure 0-72 shows the relative luciferase activity after transient transfection experiments (incubation with 100 μg/mL of test substances). Trials with stably transfected cells were carried out with three concentrations of test substances (100 μg/mL, 30 μg/mL, 10 μg/mL) and the results are reported in Figure 0-73.

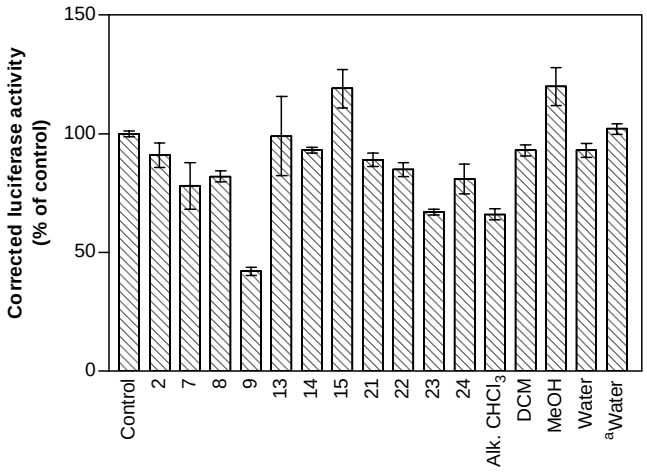


Figure 0-72

Potential regulation of NOS expression by different extracts and isolated compounds of *E. vacciniifolium*. The transient transfected EA.hy 926 cells were either kept untreated (Control) or exposed to test substances (100 μg/mL) for 12 hs. Then, the cells were lysed, and luciferase activity was determined. The relative luciferase activity (corrected with renilla-luciferase activity) was taken as a measure of NOS III promoter activity. Bars represent mean ± S.E. of three independent experiments. ([a] Tannin-free extract)

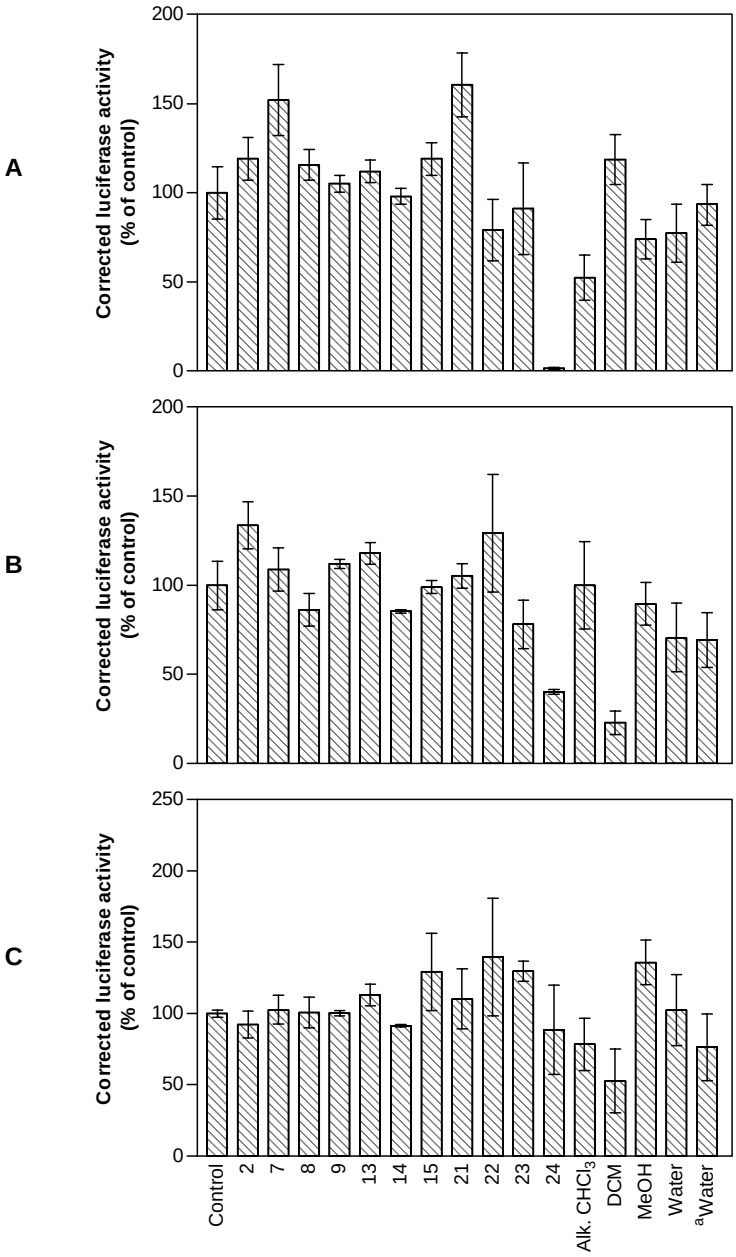


Figure 0-73

Potential regulation of NOS expression by different extracts and isolated compounds of *E. vacciniifolium*. The stable transfected EA.hy 926 cells were either kept untreated (Control) or exposed to test substances (A: 100 μg/mL; B: 30 μg/mL; C: 10 μg/mL) for 18 hs. Then, the cells were lysed, and light units determined. The relative luciferase activity (corrected with renilla-luciferase activity) was taken as a measure of NOS III promoter activity. Columns represent means ± S.E. of three independent experiments. ([a] Tannin-free extract)

In both experiments, the extracts and pure compounds showed low activity. The pGL_3-NOS III-Hu-1600 and the pNOS III-Hu-3500-Luc-neo (containing a 1.6-kb NOS III promoter fragment and a 3.5-kb human eNOS promoter fragment driving the luciferase genes, respectively) showed significant basal activity compared with the pGL_3-Basic (containing a promoterless luciferase gene) (data not shown).

In the transient transferase experiments, incubations with the test substances for 12 hs did not appreciably augment the activity of the human NOS III promoter and some compounds (*e.g.*, **9**) even inhibited it (Figure 0-72).

The stable transferase experiments showed similar results to the transient transferase ones. Incubations with different concentrations of test substances for 18 hs did not significantly increase the eNOS promoter. Compound **24** seemed to have an inhibition activity against this promoter as the corrected luciferase activity decreased with the increase of the compound concentration (Figure 0-73).

The current study did not allow to identify interesting compounds as efficacious stimulators of expression of the enzyme NOS III, implicated in vasoprotection, antiatherosclerosis and erection.

2.5.4. Cytotoxicity tests

The isolated tropane alkaloids from *E. vacciniifolium* were evaluated against a panel of human tumor cell lines according to established protocols (see chapter VI.6.7). In addition to the six cell lines used for primary cytotoxicity screening (human lung, colon, oral epidermoid and hormone-dependant prostate cancer cell lines; human telomerase reverse transcriptase-retinal pigment epithelial and human umbilical vein endothelial cells), the compounds were tested with a drug-resistant oral epidermoid cancer cell line (with and without vinblastine). For pure compounds, activity is taken as ≤ 5 μg/mL. The results of these bioassays are reported in Table 0-11.

Table 0-11

In vitro cytotoxicity of alkaloids **2**, **4**, **7**-**9**, **11**-**15** and **20**-**24** in a human tumor panel.[a]

	ED_{50} (μg/mL)[b]							
Compd	Lu1	Col2	KB	LNCaP	hTERT-RPE1	HUVEC	KB-V1 (-)	KB-V1 (+)
2	>20	>20	>20	>20	>20	>20	>20	>20
4	9.4	>20	>20	>20	>20	>20	>20	5.6
7	>20	>20	>20	>20	>20	>20	>20	>20
8	>20	>20	>20	>20	>20	>20	>20	>20
9	15.1	>20	>20	>20	>20	>20	17.8	4.7
11	>20	>20	>20	>20	>20	>20	>20	>20
12	>20	>20	>20	>20	>20	>20	>20	>20
13	>20	>20	>20	>20	>20	>20	>20	>20
14	>20	>20	>20	>20	>20	>20	>20	>20
15	>20	>20	>20	>20	>20	>20	>20	>20
20	>20	>20	>20	>20	>20	>20	>20	16.8
21	4.5	16.0	6.2	9.8	>20	>20	5.7	2.6
22	>20	>20	>20	>20	>20	>20	>20	4.1
23	>20	>20	>20	>20	>20	>20	13.6	6.0
24	4.4	12.8	6.9	5.7	>20	>20	5.9	1.9

[a] Key to human cancer cell lines used: Lu1, lung cancer; Col2, colon cancer; KB, oral epidermoid carcinoma; LNCaP, hormone-dependant prostate cancer; hTERT-RPE1, human telomerase reverse transcriptase-retinal pigment epithelial cells; HUVEC, human umbilical vein endothelial cells; KB-V (-), drug-resistant KB assessed in the absence of vinblastine (VLB); KB-V (+), drug-resistant KB assessed in the presence of VLB (1 μg/mL).

[b] The concentration of pure compound required to produce a response in 50% of the subjects to whom the compound was given. Also known as median effective dose.

As deduced from Table 0-11, none of the isolated compounds demonstrated appreciable activity against Lu1, Col2, KB and LNCaP cells in culture (>5 μg/mL). None of the tropane alkaloids seemed to have a non-specific toxicity against hTERT-RPE1 and HUVEC cells.

Compounds **21** and **24** exhibited both a certain activity and selectivity for the multidrug-resistant KB-V1 cell line in the presence of vinblastine (ED_{50}, 2.6 and 1.9 μg/mL, respectively), but they did not approach the potency (<1 μg/mL) or selectivity of tropane alkaloids from *E. pervillei* Baill. (Silva *et al.*, 2001). Moreover, these two compounds showed a modest cytotoxicity with Lu1 (4.5 and 4.4 μg/mL, respectively) and KB (6.2 and 6.9 μg/mL, respectively) cells. Compounds **4**, **9** and **22** displayed low potency against KB-V1 cells assessed in presence of VLB, but were more selective than **21** and **24** (Table 0-12). In the absence of VLB, **4** and **22** did not show any activity against KB-V1 cells (>20 μg/mL).

However, when VLB was added to the media in the presence of these two compounds, chemosensitivity was partially restored (5.6 and 4.1 μg/mL, respectively).

Table 0-12

MDR-reversing activity of compounds **4**, **9**, **21**-**24** and pervilleines A and B.

Compound	KB[a]	KB-V1 (-)[a,b]	KB-V1 (+)[a,b]	ED_{50}[KB-V1 (-)]/ED_{50}[KB-V1 (+)]
4	>20	>20	5.6	>3.6
9	>20	17.8	4.7	3.8
21	6.2	5.7	2.6	2.2
22	>20	>20	4.1	>4.9
23	>20	13.6	6.0	2.3
24	6.9	5.9	1.9	3.1
Pervilleine A	>20	>20	0.3	>66.7
Pervilleine B	>20	8.8	0.1	88

[a] Results are expressed as ED_{50} values (concentration of pure compound required to produce a response in 50% of the subjects to whom the compound was given) in μg/mL.
[b] Incubations were performed in the presence (+) (1 μg/mL) or absence of VLB.

A multidrug-resistance inhibition by several isolated alkaloids was demonstrated in these cytotoxicity tests, even though the potency was approximately one order of magnitude lower than those of pervilleines A and B.

Some observations concerning the structural requirements for activity within these tropane alkaloids may be made from the cytotoxicity panel data of the isolated compounds shown in Table 0-11. When these alkaloids are considered specifically, two ester substituents at C-3 and C-6 were seen to be essential to obtain an activity against KB-V1 cells in the presence of VLB. The tropane moiety can be substituted at both positions by a 1-methyl-1*H*-pyrrole-2-carboxylic or a 1*H*-pyrrole-2-carboxylic acid, and at C-3 by a 4-hydroxy-3,4-dimethoxybenzoic acid. The absence or presence of a hydroxyl group at C-7 led to approximately the same cytotoxic activity for KB-V1 cells in the presence of VLB, but sometimes reduced or increased selectivity.

Although the results of these preliminary cytotoxicity tests were not significant, some of the data provide encouragement for further work. It would be reasonable to isolate similar new compounds and make some derivatives of the most active ones in order to evaluate their cytotoxicity and selectivity.

V. Conclusions and perspectives

1. Qualitative and quantitative determination of yohimbine in authentic yohimbe bark and commercial aphrodisiacs

The first part of the present work, consisting of the evaluation of the potential risks of commercialized aphrodisiacs on consumer health, has given interesting and, in some ways, surprising results. The amount of yohimbine measured and expressed as the minimal dose per day appearing on the product labels has a relative large range, from a minimum of 1.32 to a maximum of 23.16 mg. Even though the therapeutic index of yohimbine is not excessively narrow, this difference in the amount of active principle in the aphrodisiac products is alarming.

In view of the qualitative and quantitative results obtained, we must consider natural aphrodisiac products as a potential cause of toxicity. This toxicity may be due to the product itself, to an interaction with other products or to contamination of the product. In addition, the increase in the last decade of herbal medication assumed to be safe by patients could amplify the potential health hazards to the community. The high costs of modern medicine have driven some patients to seek self-medication to reduce the costs, with all the consequences. Many of these products are commercialized as dietary supplements, evading any quality assessment and omitting specific indications. Rigid quality control standards are not required for nutraceuticals, leading to substantial variability in the purity and the potency of these substances (LaFrance *et al.*, 2000). Absence of real directives about these herbal aphrodisiacs has proved to be very dangerous, as substitution of components, adulteration with pharmaceuticals, incorrect preparation of crude plant material, and even plant confusion have yielded cases of toxicity in herbal medicine (Ioset *et al.*, 2003).

In the context of a more restricted regulation of the herbal medication market, the LC-UV-APCI-MS^n technique has proved to be a selective, sensitive, robust and relatively rapid method to detect and quantify yohimbine in a very broad survey of aphrodisiac products. In the absence of certain isomers such as corynanthine, both LC-APCI-MS and LC-ESI-MS have proved to be very selective and robust methods with sensitivities 20 times higher than the LC-DAD-UV method. The advantage of the LC-DAD-UV technique compared to the LC-API-MS is its larger linearity range and the possibility to quantify yohimbine in products containing its isomers in small quantities (less than 1% of the total yohimbine amount). On the other hand, LC-API-MS techniques could be helpful for the detection of yohimbine epimers in authentic yohimbe bark or complex mixtures, because of their stereochemical ionization discrimination. The linearity range has also proved to be important for the standardization of the sample preparation of a large variety of aphrodisiac products with different dosage forms. The relatively high standard deviations obtained for certain products

analyzed were the result of the non-homogeneity of these products and the presence of other substances and excipients that normally are not present in plant material. In order to avoid this problem we could have used a specific extraction method adapted for each product, but this would counter the aim of our study, namely the development of a standard method for the detection and quantification of yohimbine in aphrodisiac products.

To conclude, although many products sold over the counter or on internet are labeled as "natural", this does not ensure the product's safety or efficacy. The research undertaken here is aimed to be of benefit to patients who employ this kind of self-medication.

2. Phytochemical investigation of *Erythroxylum vacciniifolium* Mart.

The second part of this work consisted in the phytochemical and pharmacological investigation of *Erythroxylum vacciniifolium* Mart. (Erythroxylaceae), a plant used in Brazilian traditional medicine as an aphrodisiac. The preliminary information on the structures of the compounds present in the alkaloid extract were obtained on-line using different coupled techniques: high performance liquid chromatography (HPLC) coupled to diode array UV detection (LC-UV-DAD), to mass spectrometry (LC-MS) and to nuclear magnetic resonance spectroscopy (LC-NMR). All the data obtained by these LC-hyphenated techniques were processed separately or in partial combination to achieve structural identification of the compounds in the extract. A complete hyphenation of all these techniques together could be problematic because of the differences in the sensitivities of the detectors (Louden *et al.*, 2001). The dereplication of crude plant extracts with LC-hyphenated techniques represents a strategic element to avoid finding known constituents and to target the isolation of new bioactive products, rendering the investigation of new bioactive natural products more rapid and efficient.

Preliminary analyses showed homogeneity in the spectral data recorded for all constituents of the alkaloid extract, suggesting close structural similarities. The on-line methods presented in the present work allowed complete or partial identification of 24 tropane alkaloids of *E. vacciniifolium* Mart.. The interpretation of the data permitted the structural elucidation of 6 new tropane alkaloids (**7**, **13** and **21**-**24**). The structures of the other 18 detected compounds were partially characterized as di- or trisubstitued tropane alkaloids, mono- or diesterified by pyrrolic, methylpyrrolic, hydroxydimethoxybenzoic or hydroxyphenylacetic acids, and they were found to be potentially new natural products. Some of these compounds (**1**, **3**, **5**, **10** and **16**-**19**) were detected as tropane alkaloid *N*-oxides. In order to completely elucidate their structures and to perform a screening of their biological activities, a targeted isolation of the constituents of the alkaloid extract was undertaken.

The main components of the enriched alkaloid extract were purified by medium pressure liquid chromatography (MPLC) and semi-preparative high performance liquid chromatography (SP-HPLC) to yield 17 new tropane alkaloids (catuabines E-I, their derivatives and vaccinines A and B).

All catuabines isolated during this and previous studies are tropane alkaloids di- or trioxygenated at 3-, 6- or 7-positions, mono- or diesterified with 1*H*-pyrrole-2-carboxylic, 1-methyl-1*H*-pyrrole-2-carboxylic, 4-hydroxy-3,5-dimethoxybenzoic, 3-hydroxyphenylacetic or acetic acids. One of the isolated compounds (**18**) was identified as a tropane alkaloid *N*-oxide (catuabine E *N*-oxide). The occurrence of tropane alkaloid *N*-oxides in plants has previously been reported (Aripova, 1996; Katavic *et al.*, 1999; Phillipson and Handa, 1978; Silva *et al.*, 2001). But, according to a study by Achenbach and co-workers which reported the *N*-oxidation of indole alkaloids occurring in the presence of air or during work-up of plant extracts (Achenbach *et al.*, 1997), it is not possible to conclude whether the *N*-oxides detected in *E. vacciniifolium* Mart. are genuine natural products or artifacts.

The isolated tropane alkaloids are interesting for their ester moieties, which are unique to the species of the studied genus, *E. vacciniifolium*. The tropane moieties of alkaloids do not show intrageneric chemotaxonomic characteristics, but esterifying acids are often distinguishing features of certain species (El-Iman *et al.*, 1987; Evans, 1981; Griffin and Lin, 2000). Pyrrole-2-carboxylic and 1-methyl-pyrrole-2-carboxylic acid have been found only in this species (section Archerythroxylum) (Graf and Lude, 1977, 1978), while the 3-hydroxyphenylacetyl moiety found in alkaloid **2** (catuabine G) has been reported in two different species, *E. hypericifolium* Lam. and *E. pervillei* Baill., both belonging to the section Venelia (Al-Said *et al.*, 1986a; Silva *et al.*, 2001). Although the 3,4,5-trimethoxybenzoic acid substituent is very common in the *Erythroxylum* genus, the 4-hydroxy-3,5-dimethoxybenzoyl unit found in compounds **4** (7β-hydroxycatuabine F) and **9** (catuabine F) has to be considered unique for the time being.

Vaccinines A and B introduce a new series of tropane alkaloids as they are dioxygenated at 3-, 4- or 6-positions. The esterifying unit of vaccinines, the same as for catuabines, is placed at C-4, while the hydroxyl group can be at C-3 or C-6 positions. Tropanes with esters at C-2 or C-4 positions are rare in nature, whereas carboxyl groups (*e.g.* ecgonine derivatives) and hydroxyl groups (*e.g.* calystegines) at the same locations are common. In conclusion, this study illustrates that *E. vacciniifolium* Mart. is a source of original tropane alkaloids, unique for their esterifying acids and ester location, representing a useful contribution to the chemotaxonomy of the genus.

In order to evaluate their potential therapeutic interest, the crude extracts of the bark of *E. vacciniifolium* Mart. were examined in different bioassays. They were submitted to four TLC bioautographic assays to evaluate their potential bioactivities, including antifungal activities

against *Cladosporium cucumerinum* and *Candida albicans*, antibacterial activity against *Bacillus subtilis*, and acetylcholinesterase inhibitory activity. The enriched alkaloid extract exhibited a weak AChE inhibitory activity, while none of the extracts showed either antifungal or antibacterial activity. Based on this positive result, the isolated compounds were individually tested for this inhibitory activity. Only compound **24** (catuabine E) showed a certain activity which was clearly noticeable down to 1 μg, even though this value was much lower than the positive standard, galanthamine (active down to 0.01 μg).

With the aim of discovering new compounds for the treatment of erectile dysfunction, the crude extracts of *E. vacciniifolium* Mart. were evaluated for their specific inhibitory activity against PDE2 and PDE5. The extracts showed only weak inhibitory activities against the two isoenzymes and none of the results met significance criteria at the concentrations used. Thus, from these primary assays, the aphrodisiac character of *E. vacciniifolium* cannot be assigned to the phosphodiesterase inhibitory activity, but it has probably a different pharmacological origin.

Since nitric oxide (NO) is one of the most important neurohumoral factors involved in erection (Archer, 2002), the potential regulation of nitric oxide synthase expression by different extracts and isolated compounds of *E. vacciniifolium* Mart. was evaluated. Unfortunately, these bioassays did not allow the identification of lead compounds as efficacious stimulators of expression of the enzyme NOS III, implicated in vasoprotection, antiatherosclerosis and erection.

In our ongoing search for new bioactive compounds from higher plants, the alkaloid extract and new tropane alkaloids isolated during this work were also evaluated against a panel of human tumor cell lines, as recent reports indicated that some new tropane alkaloids from different species of *Erythroxylum* resulted in extremely potent inhibitors of multidrug-resistant tumor cells (Mi *et al.*, 2001; Mi *et al.*, 2002b).

Several isolated alkaloids demonstrated a certain bioactivity in these cytotoxicity tests, even though their potency or selectivity did not approach those of pervilleines A and B, two tropane alkaloids purified from *E. pervillei* Baill. (Silva *et al.*, 2001). Although the results of these preliminary cytotoxicity tests were not significant, some of the data provide encouragement for further work.

3. Perspectives

As an extension of this work, the development of new methods for the detection and quantification of other dangerous or toxic active principles, *e.g.* cantharidin, in commercialized aphrodisiacs could be considered. Many of these herbal products are still sold as nutritional supplements with no state or other approval and without making "official" therapeutic claims. Consumers should also be made aware that such preparations may not have the composition that is declared on the label.

Although the alkaloid extract of *E. vacciniifolium* Mart. was intensively investigated, there are still several minor compounds to isolate from it. After the LC-APCI-MS analysis of the polar fractions of the first MPLC separation, which showed the presence of new tropane alkaloids, purification of these fractions has been undertaken. Preliminary investigations have allowed a partial elucidation of these compounds as tropane or nortropane alkaloids di- or trioxygenated at 3-, 6- or 7-positions, monoesterified by 1-methyl-1*H*-pyrrole-2-carboxylic acid. Certain of these molecules seem to be alkaloid *N*-oxides. The complete determination structure with relative and absolute configurations of this new series of tropane alkaloids is underway. In order to discover a therapeutic potential for these compounds, they will also be submitted to all bioassays described in this work.

In view of the results obtained during the phytochemical investigation of *E. vacciniifolium* Mart., new biological tests on the isolated compounds are needed to find a basis for the traditional use of this plant as an aphrodisiac.

A valuable experimental tool for investigating local erectile responses to different pharmacological agents could be a simplified *in vitro* preparation of the rat corpus cavernosum, the anatomy of which is very close to that of man (Italiano *et al.*, 1994). This test, compared with the *in vitro* enzymatic assays, has the advantage of measuring simultaneously the activity of the compounds on different biological targets involved in erection function, *e.g.* α-adrenergic receptors (De Tejada *et al.*, 1999).

Since sexual function is also bound to different neuro-biological areas in the central nervous system, an investigation of the activity of the new compounds on animal models could give significant results. A standard test to measure the effects of drugs on sexual activity is the evaluation of copulatory behavior in male rats. Components of sexual behavior in male rats can be categorized as motivational (libido; sexual arousal) or consummatory (potency; erectile and ejaculatory ability) (Bitran and Hull, 1987). In general, the mating test consists in recording and comparing the occurrence of each mount, intromission, and ejaculation in vehicle control group and in compound treated group. Sexually receptive females of the same strain serve as sexual partners and the frequency of pregnancy could also be an indicator of

the compound activity. In addition to mount and intromission latencies serving as indices of the level of sexual arousal, the mating test can be assessed by recording the frequency at which the male mounts the female in the absence of sensory feedback from genitalia (as after penile desensitization). The measurement of sexual activity by using the mating tests could aid the discovery of the sites of action of *E. vacciniifolium* Mart. components.

As reported beforehand, some of the results obtained with cytotoxicity tests open several new perspectives. The isolation of a series of compounds having the same basic skeleton could be helpful for the investigation of the structure-activity relationships (SAR) of these molecules. Drug design is an iterative process which begins with a compound that displays an interesting biological profile and ends by optimizing both the activity profile for the molecule and its chemical synthesis. Without a detailed understanding of the biochemical process(es) responsible for activity, the hypothesis generally is refined by examining structural similarities and differences for active and inactive molecules. Compounds are selected for synthesis which maximize the presence of functional groups or features believed to be responsible for activity. In this work, the most hydrophobic catuabines exhibited a certain cytotoxicity against specific tumor cell lines; it would be then reasonable to synthesize some derivatives in order to increase this activity and render it more specific. The alternative to this approach to compound optimization could be to develop a theory that quantitatively relates variations in biological activity to changes in molecular descriptors which can easily be obtained for each compound. A quantitative structure activity relationship (QSAR) can then be utilized to help guide chemical synthesis.

In recent work, the hypothesis was put forward that there is a certain similarity between the multidrug resistance phenotype of mammalian tumor cells and quinoline resistance in *Plasmodium falciparum*, the causative agent of the most deadly form of human malaria (Bray and Ward, 1998). Chloroquine has been one of the most successful drugs ever developed against this parasite, but recent evolution and spread of resistance to this substance and other quinoline-containing drugs means that these compounds are now virtually useless in many endemic areas. Since it was demonstrated that chloroquine resistance could be circumvented *in vitro* by a number of structurally and functionally unrelated compounds such as verapamil and desipramide, the future prospects for the use of quinoline compounds are considerably improved. The phenomenon of resistance reversal by these compounds is also a key feature of vinblastine resistance in mammalian tumor cells. In view of the cytotoxicity results obtained by testing the compounds isolated from *E. vacciniifolium* Mart., new assays to evaluate the capability of these compounds to reverse the resistance to quinoline-containing drugs in *P. falciparum* could be undertaken.

VI. Experimental Part

1. Materials and extraction

1.1. Analysis of commercial aphrodisiacs containing yohimbine

Materials. Authentic yohimbe bark powder was obtained from Dixa AG (St. Gallen, Switzerland). Aphrodisiac products were purchased from different sources found on the Internet (Table 0-1). Yohimbine HCl was purchased from Carl Roth GmbH (Karlsruhe, Germany). Rauwolscine hydrochloride, corynanthine hydrochloride, codeine and yohimbinic acid monohydrate were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

HPLC grade acetonitrile was obtained from Romil LTD (Cambridge, UK). De-ionized water was prepared using a Reinstwasser-System Clear cartridge system (SG GmbH, Hamburg, Germany) and passed through a 0.45 μm filter (Millipore, Bedford, MA, USA). Analysis grade triethylamine (Et_3N) was purchased from Fluka Chemie GmbH (Buchs, Switzerland). Synthesis grade chloroform (SDS SA, Peypin, France) and reagent grade ammonia solution 32% (Merck KgaA, Darmstadt, Germany) were used in the extractions. Deuterated methanol (isotopic purity of 99.50 %), purchased from Dr. Glaser AG (Basel, Switzerland), was used in the preparation of deuterium-labeled yohimbine.

Extractions. For the extraction of yohimbe, 500 mg of authentic bark powder were moistened with 1 mL of concentrated NH_4OH and extracted four times with 100 mL $CHCl_3$ during 1 hour. The chloroform layers were united, and solvent was removed by rotary evaporation (Büchi Rotavapor) at 35°C. Aphrodisiac products included solid (tablets, gelatin capsules and bulk powder) and liquid dosage forms. For the liquid preparations, a volume of 15.0 mL was evaporated to dryness at 35°C before being treated as described before. For tablets, bulk powder and content of gelatin capsules, 5000 mg were considered. They were grounded and homogenized in a mortar and pestle. The fine powders were moistened with 5 mL of concentrated NH_4OH and extracted four times with 150 mL $CHCl_3$ during 1 hour. The chloroform layers were combined and treated as described before. The quantities for the liquid and solid dosage forms corresponded to 1 to 4 times of the maximal daily doses suggested by the manufacturers. Each sample, including authentic bark powder, aphrodisiac products and spiked sample for recovery measures, were extracted in triplicate. In order to validate a quantitative extraction procedure, a fifth extraction was performed and analyzed by HPLC separately in order to determine the amount of residual alkaloids.

Table 0-1

Name, form and origin of aphrodisiac products containing yohimbine.

Sample	Name	Form	Origin
1	Vuka vuka Herbal Tea	Powder	Zim4U Trading, Canada
2	Vuka vuka	Capsules	Zim4U Trading, Canada
3	Vuka vuka	Tablets	Kejo Limited Company, Clearwater, FL, USA
4	Vuka vuka	Liquid	Kejo Limited Company, Clearwater, FL, USA
5	Extra-Plus (Vuka vuka)	Capsules	James Mobb Imm. Enhanc., Harare, Zimbabwe
6	Vuka Nkuzi	Capsules	Ageless Co., Sunnyside, RSA
7	Vuka vuka	Powder	Mbara market, Harare, Zimbabwe
8	Erectol	Capsules	Fantasy Man, Tampa, FL, USA
9	Milagro	Tablets	International Vascular Clinic, Largo, FL, USA
10	Stamina Chi	Liquid	Advanced Living Foundation, Paia, HI, USA
11	Virile 1	Tablets	Biotech Corporation , Glastonbury, CT, USA
12	Virile-max (Male formula)	Capsules	Capital Health products, Boca Raton, FL, USA
13	Super Herbal V	Capsules	M.D. Healthline, Phoenix, AZ, USA
14	Male-Repro	Masse	Ayurvedic Rasayanas Intl., Eugene, OR, USA
15	Endow Plus (for men)	Capsules	Tiger-one.com, Dewey, AZ, USA
16	Herbal V	Tablets	Smart Health USA, Inc., Bevverly Hills, CA, USA
17	Virility-V (Herbs for Life)	Tablets	Herbs for Life, Salt Lake City, UT, USA
18	UVP - Male formula	Tablets	NuBio Research, Inc., Cincinnati, OH, USA
19	Bois bandé des caraïbes	Liquid	Harmonie SA, Couvet, Switzerland
20	Kama Sutra	Tablets	P.M.C., GmbH, Monschau, Germany

1.2. “Catuaba”

The stem bark of *Erythroxylum vacciniifolium* was collected in Buraquinho rain forest (João Pessoa, Paraíba, Brazil), in August 2000. A voucher specimen was deposited at the HLPQN (Herbarium do Laboratorio de Química de Produtos Naturais), Universidade Federal de Paraíba, 58059 João Pessoa, Paraíba, Brazil (JPB.-N° 152) and identified by Prof. Zoraide Maria de Medeiros Gouveia of the Department of Science of Nature, University of Paraíba, Brazil and Dr. Douglas C. Daly, The New York Botanical Garden.

The collected plant material was air dried and ground in liquid nitrogen to prevent heating that could alter its components. The pulverized stem bark (840 g) was moistened with 20 mL of concentrated NH_4OH and exhaustively extracted with 3L of $CHCl_3$ three times during 24 hours at room temperature and under constant agitation. After filtration of the extracts, $CHCl_3$ was

removed by rotary evaporation (Rotavapor Büchi) under vacuum at a maximum temperature of 35°C. The filtrate was neutralized with concentrated HCl and was successively extracted with about 3L dichloromethane and then 3L methanol. Maceration was performed 3 times for each solvent during 24 hours at room temperature and under continuous agitation. Solvents were evaporated to dryness as described before. A water extract was carried out in a similar way, with a second sample of ground material (16.5 g) exhaustively extracted with 0.5L of water three times during 24 hours. Another water extract was performed with the same amount of pulverized stem bark (16.5 g) infused in 0.5L boiling water during 1 hour. The water extracts were respectively combined and lyophilized. Table 0-2 presents the different extraction conditions and the weights of extracts obtained for *E. vacciniifolium*.

Table 0-2

Extraction conditions and corresponding amount of extract obtained for *E. vacciniifolium* Mart.

Extracts	Plant material	Solvent		Time	Temperature	Extract
Alk. $CHCl_3$	840 g	3 x 3L	$CHCl_3$	3 x 24 hs	R.T.	15.74 g
DCM	840 g	3 x 3L	DCM	3 x 24 hs	R.T.	5.92 g
MeOH	840 g	3 x 3L	MeOH	3 x 24 hs	R.T.	81.83 g
Water	16.5 g	3 x 0.5L	H_2O	3 x 24 hs	R.T.	2.93 g
Water	16.5 g	0.5L	H_2O	1 hs	100°C	4.58 g

2. Analytical chromatographic methods

2.1. Thin layer chromatography (TLC)

Thin layer chromatography (TLC) has so far been applied more than any other chromatographic technique and has become widely adopted for rapid and positive analysis of drugs and drug preparations. The popularity of this method is due to three main reasons: the time required for the identification of most characteristic constituents of a drug by TLC is very short, TLC gives semi-quantitative information on the main constituents of the drug or drug preparation and the chromatographic drug fingerprint provided by TLC is suitable for monitoring the identity and purity of drugs, and for detecting adulterations and substitutions (Wagner and Bladt, 1996).

TLC is the method of choice for routine phytochemical analyses of crude extracts, fractions and pure compounds. This technique is also used as a support for chemical and biological screening of extracts and pure compounds.

Commercially available TLC plates were used: Silicagel 60 F_{254} pre-coated TLC aluminium sheets (Merck, Darmstadt). Some biological tests (*Candida albicans* and *Bacillus subtilis*) were performed on Silicagel 60 F_{254} pre-coated TLC glass plates (Merck, Darmstadt). The TLC plates were developed in twin trough Camag chromatographic tanks saturated with the appropriate eluent. The solvent systems, often consisting of a binary or tertiary mixture, were adapted to the specific needs of an analysis. They were chosen for their minimal temperature sensitivity and all TLC separations were performed between 18 and 24°C. In order to obtain sharply resolved zones, the quantity of material applied to the chromatogram was usually 50-100 μg of extracts and 5-10 μg of pure compounds. After migration, the detection of the main, characteristic compounds of a drug, was carried out by the observation of the extinguishing of fluorescence in UV-254 nm, the appearance of fluorescence in UV-366 nm and the coming out of striking colors after revelation with appropriate chemical reagents. General TLC conditions for extracts and fractions were resumed in Table 0-3.

Table 0-3

Standard TLC conditions employed systematically for extracts and fractions separation (Silicagel 60 F_{254} pre-coated TLC aluminium sheets from Merck, Darmstadt).

Extracts or fractions	Solvent system	Detection
Alkaline $CHCl_3$ extract	toluene-ethyl acetate-diethylamine (70:20:10)	Dragendorff reagent
DCM extract	hexane-ethyl acetate (1:1)	Godin's reagent
MeOH extract	chloroform-methanol-water (65:35:5)	Godin's reagent
Water extract	chloroform-methanol-water (4:5:2)	Godin's reagent
Polar fractions	chloroform-methanol-water (65:35:5)	Godin's reagent
Hydrophobe fractions	hexane-ethyl acetate (1:1)	Godin's reagent
Alkaline fractions	toluene-ethyl acetate-diethylamine (70:20:10)	Dragendorff reagent

2.2. High performance liquid chromatography coupled to ultraviolet detection (HPLC-DAD-UV)

High performance liquid chromatography (HPLC) is one of the most appreciated techniques in modern phytochemical analysis. The separation of complex mixtures is based on the selective distribution of analytes between a liquid phase and a stationary phase, as for TLC method. The advantage of HPLC in relation to TLC is the separation resolution, the sensible decrease of the detection limit and the possibility of automation of the procedure. A conventional HPLC system is based on the injection of the sample by means of an injection port into the mobile phase stream delivered by the high-pressure pump and transported through the column, the heart

of the system where the separation takes place. The separation is monitored with a flow-through detector and visualized by an interface (integrator or computer) (Niessen, 1999). Currently, one of the most utilized detectors coupled to HPLC is the diode array detector (DAD). The development of DAD in early 1980s made a significant change in relation to classical UV detectors. In fact, the DAD adds a third dimension (the wavelength) to the time and absorption, giving a wealth of information, for example, on peak purity and identity (Huber and George, 1993).

In this study, analytical HPLC was used to: analyze qualitatively and quantitatively the extracts of commercial aphrodisiacs, analyze raw extracts and fractions of "catuaba", guide the separation and isolation processes, optimize the conditions for MPLC and semi-preparative HPLC, and check the purity of the isolated compounds.

Two HPLC systems were used for the different analyses: a HP-1100 system (Hewlett Packard, Palo Alto, CA, USA) equipped with a binary pump, with a DAD and an autosampler; a HP-1090 series II system (Hewlett Packard, Palo Alto, CA, USA) including a quaternary pump, DAD and an autosampler. The various components of these two HPLC systems were controlled by Agilent ChemStation 8.01 software. This software was also employed for the data processing. The HPLC conditions routinely used for the analysis of commercial aphrodisiacs and "catuaba" were reported in Table 0-4. In some cases, these conditions were adapted to the specific requirements of the analyses.

Table 0-4

General HPLC conditions for the analysis of commercial aphrodisiacs and "catuaba".

	Commercial aphrodisiac analysis	**"Catuaba" analysis**
Column	Nucleosil 100-5 C_{18} AB (Macherey-Nagel, Düren, Germany)	Nucleodur 100-5 C_{18} (Macherey-Nagel, Düren, Germany)
Column size	125 x 2.0 mm i.d., 5 μm	125 x 4.6 mm i.d., 5 μm
Pre-column size	8 x 3.0 mm i.d., 5 μm	8 x 4.0 mm i.d., 5 μm
Solvent system	MeCN (+2 mM Et_3N) : water (+2 mM Et_3N)	MeCN (+2 mM Et_3N) : water (+2 mM Et_3N)
Gradient	20:80 to 100:0 in 20 min (4 elution steps)	5:95 to 100:0 in 34 min (5 isocratic elution steps)
pH	11	11
Pressure	40-100 bar	45-110 bar
Temperature	20-21°C	19-24°C
Flow rate	0.3 mL/min	1.0 mL/min
Detection (DAD)	280 nm	272 nm (UV scan: 200-500 nm)

2.3. High performance liquid chromatography coupled with mass spectrometry (LC-MS)

The combination of high performance liquid chromatography - the abbreviation LC is used throughout this text - and mass spectrometry (MS) offers the possibility of taking advantage of both LC as a powerful separation technique and MS as a powerful and sensitive detection and identification technique (Niessen, 1999). In general, MS detection is used in combination with DAD detection resulting in an extremely powerful analytical tool, which can be considered as a hyphenated technique.

LC-UV-MS was employed for the qualitative and quantitative analysis of yohimbine in commercial aphrodisiacs, for a preliminary study of "catuaba" crude extracts, for the dereplication of the "catuaba" alkaloid extract and to on-line obtain spectroscopic information.

Current LC-UV-MS analyses were made on the following apparatus and the ionization conditions are reported in Table 0-5:

- HPLC HP-1100 (see Ch. 2.2);
- Atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) interface;
- Finnigan MAT (San José, CA, USA) LCQ ion trap or TSQ triple quadrupole mass spectrometer, or Micromass (Manchester, UK) LCT time-of-flight mass spectrometer.

Table 0-5

MS conditions for the different instruments and analyses.

	Commercial aphrodisiac analysis		**"Catuaba" analysis**	
	LCQ	*TSQ 700*	*LCQ*	*LCT*
APCI source				
Capillary temperature	150°C	150°C	150°C	-
Source temperature	-	-	-	120°C
Vaporizer temperature	360°C	360°C	380°C	500°C
Ionization mode	positive	positive	positive	positive
Corona needle current	6.0 μA	6.0 μA	6.0 μA	5.0 μA
Sheath gas pressure	90 psi (N_2)	90 psi (N_2)	60 psi (N_2)	60 psi (N_2)
In-source CID	-	-15 eV	-	-30 eV
Collision energy	30%[a]	-	35%[a]	-
ESI source				
Capillary temperature	270°C	270°C		
Source voltage	4.5 kV	4.5 kV		
Ionization mode	positive	positive		
Sheath gas pressure	90 psi (N_2)	90 psi (N_2)		
Auxiliary gas pressure	25 psi (N_2)	25 psi (N_2)		
In-source CID	-	-15 eV		
Collision energy	30%[a]	-		

[a] Energy levels on Finnigan IT-MS are given in % and not in eV since the voltages applied vary according to the *m/z* value of the precursor ions.

2.4. High performance liquid chromatography coupled with nuclear magnetic resonance (LC-NMR)

The coupling of high performance liquid chromatography with nuclear magnetic resonance spectroscopy (LC-NMR) is one of the most powerful methods for the separation and structural elucidation of unknown compounds in mixtures (Wolfender *et al.*, 2001). LC-NMR represents a potentially interesting complementary technique to LC-UV-MS in phytochemical analysis for the detailed on-line structural analysis of natural products (Wolfender *et al.*, 1998b; Wolfender *et al.*, 1998a).

The analyses were performed with following equipment:

- a Varian (Palo Alto, CA, USA) modular HPLC system, including a Varian 9012 pump, a Valco injection valve and a Varian 9050 UV detector, controlled by Varian LC Star software;
- a Varian Unity Inova 500 MHz NMR instrument equipped with a $^{1}H[^{13}C]$ pulse field gradient indirect detection microflow LC-NMR probe (flow cell 60 μL; 3 mm i.d.);
- a Valco (Houston, TX, USA) stop-flow valve E60-220.

The separations were performed by using a Nucleosil 100-5 C_{18} AB prepacked column (125 x 8.0 mm i.d., 5 μm; Macherey-Nagel, Düren, Germany) with MeCN (+ 2 mM ND_3OD) : D_2O (+ 2 mM ND_3OD) (5:95 to 100:0; 80 min). The resulting pH of the eluent was 10. In order to achieve satisfactory on-flow LC-NMR detection the amount of extract injected was increased to 3.0 mg on-column. The flow rate was increased to 1.2 mL/min and the on-flow run consisted of 107 increments of 32 transients each. The total analysis time for this experiment was 80 min. During LC-NMR the UV traces were measured at 272 nm for monitoring the chromatographic separation. For the stop-flow experiments, the UV detector was used to trigger the stop-flow valve and trap precisely the LC-peak of interest in the LC-NMR flow cell. In the stop-flow mode, 1024 transients were accumulated for each spectrum. References of the solvent signals were set at δ 2.10 for acetonitrile. For each increment, solvent suppression was performed with the WET sequence (Smallcombe *et al.*, 1995). During gradient elution the shapes of the selective pulses were automatically calculated on the fly based on a scout scan recorded before each increment.

3. Preparative chromatographic methods

3.1. Medium pressure liquid chromatography (MPLC)

The principle of this method is similar to HPLC, but it allows the separation of larger sample loads with good resolution. The equipment used consisted of a Büchi B-681 pump, a Büchi B-687 gradient device, a Knauer K 2001 UV detector, a Pharmacia LKB Rec 1 recorder and an automatic Büchi B-684 fraction collector. A Lichroprep® C_{18} stationary phase (15-25 μm, Merck) was used for all separations and it was packed in a pressure-resistant column of variable size, depending on the sample amount. The choice of the solvent system was performed by analytical LC-UV and transposed directly to MPLC. For the isolation of alkaloids, a gradient mixture of MeCN-water-Et_3N (2 mM) as mobile phase was employed. The maximal pressure was set to 20 bars and the sample was placed in an introduction cartridge after having been mixed with 2-3 times its weight of stationary phase.

3.2. Semi-preparative high performance liquid chromatography (SP-HPLC)

The term “semi-preparative” is conventionally applied to columns with an i.d. of 8-10 mm, often packed with 5-10 µm particles , and useful for the separation of 1 mg to 100 mg mixtures (Hostettmann *et al.*, 1998).

SP-HPLC was used in order to purify the compounds previously isolated by MPLC. A Nucleosil 100-5 C_{18} AB prepacked column (125 x 8.0 mm i.d., 5 μm; Macherey-Nagel, Düren, Germany) was employed for the separations.

Depending on the needs, this column was connected to two different systems. For the purification of small amounts, a Shimadzu LC-10AD pump (Kyoto, Japan) equipped with a Pharmacia LKB UV detector and a Pharmacia LKB Rec 1 recorder was used. The flow-rate was set between 1 and 2 mL/min and the injected amounts ranged from 0.3 to 1.0 mg.

For the isolation of larger quantity, a Gilson pump (model 321; Middleton, WI, USA) equipped with a Gilson UV/VIS detector (model 151) and a Gilson fraction collector (model 206) were used. All these components were controlled by Gilson Unipoint 3.2 software. The flow-rate was generally augmented to 2 mL/min and the injected amounts to 3.0 mg.

Optimization of separation conditions were determined by analytical HPLC using a Nucleosil 100-5 C_{18} AB column (125 x 4.6 mm i.d., 5 μm; Macherey-Nagel, Düren, Germany) before transposition to a semi-preparative scale.

4. Physico-chemical methods

4.1. Optical rotation ($[\alpha]^D$)

The optical rotation of the pure compounds was measured on a Perkin-Elmer 241 polarimeter (Wellesley, MA, USA) with the sodium D line (589.3 nm) as the source of light. The measurements were made at 20°C in a 10 cm long cell. The results were calculated with the following formula and the concentrations were expressed in g/100 mL, according to conventions:

$$[\alpha]_D^{20°C} = \frac{1000 \cdot \alpha}{l \cdot c}$$

α = observed rotation

l = cell length in dm

c = concentration in g/L

4.2. Circular dichroism (CD) spectroscopy

Circular dichroism (CD) spectroscopy is a form of light absorption spectroscopy that measures the difference in absorbance of right- and left-circularly polarized light by a substance.

CD spectra were recorded on a JASCO J-810 spectropolarimeter (Easton, MD, USA) in the 195-350 nm range with compound concentrations of 0.325-1.432 mM in MeOH and/ or MeCN solution. All CD spectra are reported as the difference in molar absorptivity ($\Delta\varepsilon = \varepsilon_l - \varepsilon_r$). Experiments were performed at room temperature (near 22°C) in the Department of Organic Chemistry of Prof. Sándor Antus in the University of Debrecen (Debrecen , Hungary) by Dr. Timbor Kurtán.

4.3. Infrared spectrophotometry (IR)

The IR spectra of the pure compounds were obtained on a Perkin-Elmer 1600 FTIR instrument (Wellesley, MA, USA). The technique used for the preparation of the solid sample was the formation of KBr pellets. Generally 1 mg of the isolated compound was mixed with 200 mg of dry KBr. This mixture was finely ground using a ball mill, and pressed to a transparent pellet using an evacuable die. The spectra of the pellets were recorded versus the empty reference beam (4 scans, 2.0 cm^{-1}, 500-4000 cm^{-1}).

4.4. Ultraviolet spectrophotometry (UV)

The UV spectra of the isolated compounds were recorded in ethanol on a Perkin-Elmer Lambda 20 spectrophotometer (Wellesley, MA, USA). Quartz cells were used for samples and blanks. The results were reported in λ_{max}^{EtOH} nm (log ε), where λ_{max}^{EtOH} nm represented the wave-length of the maxima and ε the extinction coefficient at this wave-length according to the Lambert-Beer law:

$A = \varepsilon \cdot c \cdot b$

A = absorbance (au)

c = molar concentration (mol/L)

b = thickness of the cell (cm)

4.5. Mass spectrometry (MS)

Mass spectra of the isolated compounds were run in the electron ionization (EI) and desorption chemical ionization (D/CI) modes. In EI, the analyte vapour is subjected to a bombardment of energetic electrons from a direct electrically-heated tungsten or rhenium filament. A radical cation $M^{+\bullet}$ is produced and it is called the molecular ion, since its *m/z* ratio corresponds to the molecular mass M_r of the analyte. In D/CI the ionization is based on a chemical reaction between a reagent gas ion (*i.e.*, NH_4^+) and the analyte molecule. The reagent gas ions are generated by EI and subsequent ion-molecule reactions. The protonated molecules reacts with an analyte molecule transferring the proton and generating a protonated analyte molecule, generally with low internal energy and consequently less prone to fragmentation than the molecular ion generated by EI ionization. In D/CI the combination of rapid heating and the direct interaction of vaporized molecules with reagent gas ions enables the mass analysis of many relatively non-volatile analytes (Kellner *et al.*, 1998).

EI and D/CI mass spectra of pure compounds were measured on a Finnigan-MAT/TSQ-700 triple stage quadrupole instrument with the following parameters:

- EI-MS: 70 eV applied on the filament; vaporization of the analytes by a linear increase of the temperature of the probe (50-300°C in 1 min); temperatures of the source and quadrupoles maintained at 150°C and 70°C, respectively.
- D/CI-MS: ammonia as reagent gas for measurements in the positive mode; linear increase of desorption filament temperature (50-1000°C in 1 min).

- In order to confirm the molecular formulae of the isolated compounds, high resolution mass spectra were recorded. The measurements were carried out on a Bruker FTMS 4.7T mass spectrometer (Billerica, MA, USA) in the Laboratoire de Chimie Organique of Prof. Titus Jenny in the University of Fribourg, Switzerland.

4.6. Nuclear magnetic resonance spectrometry (NMR)

The nuclear magnetic resonance spectrometry (NMR) was chosen as main analytical method for the structural elucidation of the isolated compounds. The NMR is a phenomenon which occurs when the nuclei of certain atoms are immersed in a static magnetic field and exposed to a second oscillating magnetic field. Under appropriate conditions, the analyte can absorb electromagnetic radiation in the radio frequency region at frequencies depending on the characteristic of the analyte.

The nuclei of greatest interest were mainly protons (^{1}H) and carbon-13 (^{13}C) as their resonances are the most important for the identification of natural organic molecules. The ^{1}H and ^{13}C-NMR spectra were recorded on a Varian Inova 500 MHz spectrometer (Palo Alto, CA, USA) at 499.87 and 125.70 MHz, respectively. The instrument was controlled by Varian VNMR software installed on a Sun workstation (Santa Clara, CA, USA). All NMR measurements were performed at 30°C in deuterated chloroform (Dr Glaser AG, Basel, Switzerland). In the case of polar compounds, a drop of deuterated methanol was previously added in order to facilitate the sample dissolution. The shifts are indicated in ppm, with trimethylsilane (TMS) as an internal standard for ^{1}H spectra, and the chloroform-*d* shift (77.0 ppm) as reference for ^{13}C spectra.

In order to observe homo- and heteronuclear correlations between proton and carbon atoms of the analyte, complementary two dimentional (2D) experiments were performed. For advanced and 2D spectra including gDQF-COSY, gHSQC, gHMBC and NOESY, standard pulse sequences provided in the original VNMR software were employed. DEPT spectra consisted of 90° pulse spectrum (CH signals positive) and 135° pulse spectrum (CH and CH_3 signal positive and CH_2 signals negative).

5. Chemical methods

5.1. Reagents for TLC detection

After development and mobile-phase solvent evaporation, zones were detected by various means. Colored substances may be viewed in daylight without any treatment. Colorless substances were first detected if they showed self-absorption in the short-wave UV region (254 nm) or if they could be excited to produce fluorescence by UV radiation (366 nm). Otherwise, detection was achieved by means of chemical reagents (chromogenic or fluorogenic reagents) producing colored or fluorescent zones. Two main chemical reagents were used to detect compounds of "catuaba" on TLC plates:

- Godin's reagent (Godin, 1954): general reagent. First, the plate was sprayed with a solution of equivalent volumes of 1% vanillin in ethanol and 3% perchloric acid in water. Then, the dried layer was covered with an ethanolic solution of 10% sulfuric acid and heated to about 100°C during 5 min. Colored zones were produced on a pale background.
- Dragendorff reagent (Munier, 1953): specific reagent (alkaloids). (a) 0.85 g of bismuth subnitrate and 10 g of tartaric acid were dissolved in 40 mL of water. (b) 16 g KI were dissolved in 40 mL of water. Color reagent: 5 mL of (a), 5 mL of (b), 20 g of tartaric acid and 100 mL of water were mixed extemporary and then sprayed on the plate to give orange zones on a yellow background.

5.2. Preparation of yohimbine-d_3

In order to improve the precision of yohimbine quantitative analysis, an internal standard was needed and isotopically labeled yohimbine was selected for the LC-MS experiments. Deuterium-labeled yohimbine ([methyl-^{2}H]-labeled yohimbine) was obtained by esterification of yohimbinic acid with [^{2}H]-methanol under acidic conditions (pH = 2). The reaction was reversible, but an excess of [^{2}H]-methanol favored a quantitative esterification. Yohimbinic acid monohydrate (2 g; Sigma Chemical Co., St. Louis, MO, USA) was dissolved in 50 mL of perdeuterated methanol (Dr Glaser AG, Basel, Switzerland) containing 0.1 M H_2SO_4 and refluxed for 5 h. The reaction was followed by a determination of the ratio of the original and the esterified acid in the reaction mixture by LC-MS. Once the reaction was quantitative, the solution was cooled to 20°C, neutralized with Na_2CO_3 and the solvent evaporated to dryness

under reduced pressure. The residue was poured into 50 mL of water, basified (pH = 9) and extracted 3 times with 50 mL of $CHCl_3$. The combined extracts were evaporated by rotary evaporation at 35°C and the residue was analyzed by MS-MS. The synthesized compound presented a protonated ion $[M+H]^+$ at *m/z* 358.4 and fragment ions at *m/z* 215.2, 144.3 and 226.2 corresponding to deuterium-labeled yohimbine. The yield was 1.8 g (90%).

5.3. Removal of tannins from plant extracts

Tannins are secondary metabolites widely distributed in the plant Kingdom. The reactivity of such polyphenolic compounds to proteins is established and several instances reported the interference of tannins in certain assays due primarly to their reaction with proteins of enzymes (Wall *et al.*, 1996).

The bark of *E. vacciniifolium* demonstrated to have polyphenolic compounds such as tannins and their elimination from methanolic and aqueous extracts had to be undertaken in order to avoid some interference during the bioassays.

The different extracts (1 g) were dissolved into 25% ethanolic solution (100 mL) containing 1 g of hide powder(for determination of tannic acid slightly chromated; Merck, Darmstadt, Germany), stirred at room temperature for 60 min and centrifuged. The supernatant was withdrawn and the procedure repeated . The supernatants were combined , dried and submitted to the bioassays.

6. Bioassays

6.1. Acetylcholinesterase (AChE) inhibitory activity

The enzyme acetylcholinesterase (AChE; EC 3.3.3.7) has the function of terminating the action of acetylcholine (ACh) at the junctions of the various cholinergic nerve endings with their effector organs or postsynaptic sites. Inhibitors of this enzyme cause ACh to accumulate at cholinergic receptor sites producing an increase of their stimulation. Anticholinesterase agents have therapeutic utility in the treatment of glaucoma, the facilitation of gastrointestinal and bladder motility, influencing activity at the neuromuscular junction of skeletal muscle, as desired in myasthenia gravis, and in the symptomatic treatment of Alzheimer's disease (Taylor, 1996).

All crude extracts and isolated compounds were submitted to a test developed to detect anticholinesterase agents directly on TLC plates (Marston *et al.*, 2002):

AChE from electric eel (1000U) purchased from Sigma Chemical Co. (St. Louis, MO, USA) was dissolved in 150 mL of 0.05 M Tris-hydrochloric acid buffer at pH 7.8. Bovine serum albumin (150 mg) (Merck, Darmstadt, Germany) was mixed with this solution in order to stabilize the enzyme during the bioassay. The developed and well-dried TLC plates were sprayed with the solution and incubated in humid atmosphere at 37°C for 20 min after being dried again. A freshly prepared mixture of 1 part of 1-napthyl acetate (250 mg/mL in MeOH) and 4 parts of "Fast Blue salt B" (Fluka, Buchs, Switzerland; 200 mg/ 80 mL water) was then sprayed on the incubated plates. A purple coloration appeared on the whole surface after 1-2 min. Active compounds (inhibitors of the AChE) were localized by colorless spots. In general, 50 μg of crude extract and 5 μg of pure compounds were spotted on the layer. Galanthamine obtained from Sigma Chemical Co. was used as positive control.

6.2. Inhibitory activity against Phosphodiesterase (PDE)

Cyclic nucleotide phosphodiesterases (PDE; EC 3.1.4.17) play a key role in the metabolism of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). These are high-capacity enzymes that rapidly degrade and thereby inactivate cAMP (to 5' AMP) and cGMP (to 5' GMP), respectively. Nine families of PDEs (PDE1 to PDE9) are known and they differ with respect to tissue distribution, substrate specificity, regulation by kinases, cGMP, or calcium, etc. The selective inhibitors of these isoenzymes are interesting pharmacological targets because of their diversity, the distinct tissue distribution pattern and the possibility of high throughput screening systems with heterologously expressed PDEs (Glossmann *et al.*, 1999).

In order to discover new compounds in the treatment of erectile dysfunction, we tested "catuaba" crude extracts for their inhibitory activity against phosphodiesterase. As Sildenafil (Viagra®), the most famous and studied compound for the treatment of erection dysfunction, is known to be a potent, competitive inhibitor of human PDE5, the biochemical assays were performed on the same isoenzyme. Since Sildenafil is a highly selective PDE5 inhibitor relative to its activity against PDE1 to PDE4 (Ballard *et al.*, 1998), the tests were also carried out on PDE2.

All these biochemical assays were undertaken at the Pharmacology Laboratories of the MDS Pharma Services – Taiwan Ltd. (Taipei, Taiwan) via study directors Fong-Chi Cheng and Ching-Chui Lin. The methods employed in this study have been adapted from the scientific literature to maximize reliability and reproducibility (Hidaka and Asano, 1976; Nicholson *et al.*, 1991). Reference standards were run as an integral part of each assay to ensure the validity of the results obtained. Assays on alkaloids, MeOH (treated or not for the removal of tannins and sugars) and DCM extracts were performed under conditions described in Table 0-6.

Table 0-6

Conditions for the test of inhibition activity against phosphodiesterase isoenzymes.

	PDE2 (cat. # 148000)	**PDE5 (cat. # 156000)**
Source	Human platelets (hypotonic buffer, polytron homogenization)	Human platelets (hypotonic buffer, polytron homogenization)
Substrate	25.1 μM [^{3}H]cAMP + cAMP	1.01 μM [^{3}H]cGMP + cGMP
Vehicle	1% DMSO	1% DMSO
Pre-Incubation	None	None
Incubation	20 min at 25°C	20 min at 25°C
Incubation Buffer	50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5	50 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5
Sample M. W.	300 g/mol (Assumed Weight)	300 g/mol (Assumed Weight)
Sample concentration	100 μM	100 μM
Reference Compound (IC_{50})	3-isobutyl-1-methyl-xantine (IBMX) (Historical: 30 μM; Concur. MIC: 58.9 μM)	3-isobutyl-1-methyl-xantine (IBMX) (Historical: 63 μM; Concur. MIC: 38.5 μM)
Quantitation Method	Quantitation of [^{3}H]adenosine	Quantitation of [^{3}H]guanosine
Significant Criteria	≥ 50% of max stimulation or inhibition	≥ 50% of max stimulation or inhibition

6.3. Nitric oxide (NO) induction

Although there are many neurohumoral factors involved in erection, nitric oxide (NO) is one of the most important (Archer, 2002). NO is a small inter- and intracellular signaling molecule generated by a family of enzymes termed NO synthases (NOS) through the conversion of L-arginine into citrulline (Knowles and Moncada, 1994). There are three well-characterized isoforms of NOS that are named after the tissues from which they were originally isolated: endothelial (eNOS), neural (nNOS), and inducible (iNOS). In the penis, nNOS is localized to nonadrenergic-noncholinergic nerves, whereas eNOS is found in the smooth muscle cells and endothelium. Only nNOS has been conclusively identified histochemically in the penis (at nerve terminals) (Burnett *et al.*, 1993), although the eNOS is expressed in the penile cytosol (Penson *et al.*, 1997). Despite the importance of nNOS, erectile function is preserved in mice in which nNos has been eliminated by targeted gene disruption. This is due to the compensatory up-regulation of eNOS (Archer, 2002). The essential role of NO in erection has been proved. The inhibition of NOS substantially reduces erection and is reversed by the NOS substrate, L-arginine. Evidence from genetic models, as well as experiments with pharmacological inhibitors, has shown that low amounts of NO generated by eNOS are important for normal vascular function, regulating vascular tone, and inhibiting platelet aggregation and leukocyte adhesion to vessel walls. Decreased endothelial NO production has been seen in pathophysiological

conditions such as atherosclerosis, diabetes, and hypertension. In view of the protective effects of NO, compounds that increase the NOS activity, expression, or both are of significant therapeutic interest.

In order to discover new compounds in the treatment of erectile dysfunction, vasoprotective and probably antiatherosclerotic properties, the different extracts and the isolated compounds of *E. vacciniifolium* were tested for their potential regulation of NOS expression. The bioassays were carried out at the Department of Pharmacology, Johannes Gutenberg University, Mainz, Germany under the direction of Dr. Huige Li and Prof. Dr. Ulrich Förstermann using the following two methods: transient transfection and stable tranfection.

Transient transfection and reporter gene assay. In the transient transfection experiments, human endothelial EA.hy 926 cells were transfected with a construct containing a 1.6-kb human eNOS promoter fragment driving the luciferase[**] gene (as reporter gene). The cells were then treated with plants extracts or pure compounds and luciferase activity were determined as a measure of the eNOS promoter activity.

EA.hy 926 cells were placed in six-well plates 24 hs before transfection. Transient transfection was performed with 3 μL/well SuperFect reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each well received 1 μg of plasmid DNA, *i.e.*, 0.9 μg pGl_3-Basic (Promega, Madison, WI, USA) or 0.9 μg of pGl_3-NOS III-Hu-1600 and 0.1 μg pRL-simian virus 40 (Promega). pGl_3-NOS III-Hu-1600 contains a 1.6-kb NOS III promoter fragment (-1600 to +23) cloned before the luciferase gene of pGl_3-Basic. Plasmid pRL-SV40 contains the renilla-luciferase gene driven by an SV40 promoter and was cotransfected for normalization. On the next day, the cells transfected with pGl_3-NOS III-Hu-1600 were left untreated or incubated with 100 μg/mL of pure compounds or extracts for 12 hs. Then the cells were lysed with passive lysis buffer (Promega) and luciferase/renilla-luciferase activities were measured with the Dual-Luciferase System (Promega). The luciferase activity was normalized by renilla-luciferase activity after subtraction of background.

Stable transfection and reporter gene assay. The principle of stable transfection is similar. EA.hy 926 cells were transfected with a construct containing a 3.5-kb human eNOS promoter fragment driving the luciferase gene. The construct contains additionally a neomycin resistance gene so that a selection is possible. The stable cells were treated with extracts and

[**] An enzyme from firefly tails that catalyses the production of light in the reaction between luciferin and ATP. Used by the male firefly for producing light to attract females and used in the laboratory in a chemiluminescence bioassay for ATP.

pure compounds, and the luciferase activity was analyzed for the eNOS promoter activity. Protein concentration in the cell lysates were measured for normalization.

EA.hy 926 cells were placed onto 30-mm cell culture dishes 24 hs before transfection. The cells (at ≈80% confluence) were transfected with DOTAP (*N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate) according to the manufacturer's recommendations (Boehringer-Mannheim, Mannheim, Germany); 10 μg pGL_3-Basic eNOS III-Hu-3500-Luc-neo was used. The cells were washed with culture medium 6 hs after transfection and incubated with medium for 18 hs. Then, the cells were separated to dilute them and incubated with medium containing 1 mg/mL G418 (blocks polypeptide synthesis and inhibits elongation. For use in the selection and maintenance of eucaryotic cells stably transfected with neomycin resistance genes). Single clones were selected from the pNOS III-Hu-3500-Luc-neo-transfected cells and propagated in medium containing 1 mg/mL G418. For analysis of enhancement of NOS III promoter activity, the stably transfected cells were incubated with pure compounds and extracts (100 μg/mL, 30 μg/mL, 10 μg/mL) for 18 hs. Extracts (200 μL) were prepared using the reporter lysis buffer (Promega), and luciferase activities were determined as described.

6.4. Antifungal test against *Cladosporium cucumerinum*

Cladosporium cucumerinum is a microscopic phytopathogenic fungus, attacking particularly members of the Cucurbitaceae family. Occurrence of *Cladosporium* species in the environment seems to be related to respiratory allergies in humans (Cruz *et al.*, 1997). In addition, other species could be responsible for cutaneous (Elgart, 1996) or central nervous system (Salaki *et al.*, 1984) infections.

Testing for antifungal activity was performed by direct bioautography on TLC (Homans and Fuchs, 1970):

After elution, the chromatograms were dried and sprayed with a conidial suspension of *C. cucumerinum* (prepared by Syngenta, Basel, Switzerland) in Sabouraud medium (Sabouraud Maltose Broth, Biokar Diagnostics, Beauvais, France). The plates were then incubated during 3 days at room temperature in a humid atmosphere. The fungal growth was accompanied by the production of a greenish grey pigment: active compounds appeared very clearly as white inhibition spots. The spotted amounts were 100 μg for extracts or fractions and 10 μg for pure compounds. Amphotericin B or miconazole were used as positive controls (10 μg), while chloramphenicol was spotted on the TLC plate as negative control (10 μg).

6.5. Antifungal test against *Candida albicans*

Candida albicans (Robin) Berkhout is a yeast present in the gastrointestinal, buccal and vaginal human commensal flora, but it can become pathogenic in immunodepressed patients. In last decades, increasing incidence of systemic candidiasis associated with AIDS and also that arising after treatment by immunosuppressive drugs, and the apparition of numerous resistances to the drugs available has given fresh impetus to the search for new agents against this pathogen. Since direct bioautography is not possible with yeasts such *C. albicans*, a simple and rapid agar overlay assay has been developed (Rahalison *et al.*, 1991). This contact bioautography technique relies on the transfer by a diffusion process of active compounds from the stationary phase into the agar layer containing the micro-organism. After incubation, the plate is sprayed with (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT). The fungus converts the MTT into an MTT formazan dye. Inhibition zones are observed as clear spots against a purple background.

The agar overlay method used is as follows:

A *C. albicans* strain was obtained from the Centre Hospitalier Universitaire Vaudois (CHUV, Lausanne , Switzerland). A colony of this micro-organism was put in 50 mL of Sabouraud medium (Sabouraud Dextrose Agar, Biokar Diagnostics, Beauvais, France) and kept for one night at room temperature with constant shaking. 1 mL of this solution was diluted with 50 mL of the same Sabouraud medium and shaken at room temperature during 6 hours to achieve micro-organism exponential growth. 5 mL of this solution was introduced in 50 mL fractions of malt agar (Biokar Diagnostics), prepared and maintained at 45°C. These inoculums contained then approximately 10^5 cells/mL and 20 mL were sufficient for a 10×20 cm plate. The extracts (100 μg) or the pure compounds (10 μg) were applied on a glass-backed silica gel TLC plate and developed as described in Ch. 2.1. Miconazole (10 μg) was used as positive control and chloramphenicol (10 μg) as negative control. After elution, the TLCs were dried. The liquid agar medium was spread on the plate in a layer of about 1-2 mm and it hardened while cooling. The different plates were incubated overnight at 30°C in a humid atmosphere, and then homogenously sprayed with the MTT solution (2.5 mg/mL). The micro-organism metabolized the MTT into purple formazan, and after 4 hours incubation, the inhibition zones were visualized as white spots on the purple background. Ethanol 94% was then sprayed on the plates to stop yeast growth.

6.6. Antibacterial test against *Bacillus subtilis*

Bacillus subtilis is a gram-positive, rod-shaped and endospore-forming aerobic bacterium. It is found in soil and rotting plant material and is non-pathogenic. It is one of the most studied gram-positive bacteria and only the gram-negative *Escherichia coli* and its relatives are substantially better understood. Several strains related to *B. subtilis* are used in the commercial production of extra-cellular enzymes, such as *B. amyloliquefaciens* alpha-amylase. Other strains produce insect toxins, peptide antibiotics and antifungals, some of which have been used in agricultural crop protection. Some bacteria in the genus cause disease, for example *B. anthracis* is the cause of anthrax. This micro-organism, often cited as biological weapon, is a zoonotic agent to which most mammals, especially grazing herbivores, are considered susceptible. Human infections result from contact with contaminated animals or animal products, and there are no known cases of human-to-human transmission (Dixon *et al.*, 1999).

The agar overlay method used in our laboratories was adapted from the literature (Hamburger and Cordell, 1987) and described as follows:

A *B. subtilis* strain (ATCC 6633) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). A colony of the germ was grown in a 50 mL liquid Luria-Bertani Broth (LBB): 10 g/L tryptone (Biokar Diagnostics, Beauvais, France) + 5 g/L autolytic yeast extract (Biokar Diagnostics) + 10 g/L NaCl. After one night of shaking at room temperature, 0.5 mL of this solution were employed to inoculate 50 ml of a second Luria-Bertani culture medium. This solution was agitated during 8 hours at room temperature to exponentially increase the micro-organism growth. Afterwards, 0.5 mL were introduced in fractions of 50 mL of Luria-Bertani Agar (LBA), prepared and kept at 45°C: LBB + 15 g/L bacteriological agar type A (Biokar Diagnostics). These inoculums contained then approximately 10^8 cells/mL and 20 mL were sufficient for a 10 × 20 cm plate. The extracts (100 μg) or the pure compounds (10 μg) were applied on a glass-backed silica gel TLC plate and developed as described in Ch. 2.1. Chloramphenicol (10 μg) was used as negative control. After TLC drying, the liquid agar medium was laid on the plate in a 1-2 mm layer. As for *C. albicans* (see Ch. 6.5), the plates were incubated and then sprayed with MTT. Active compounds could be detected as white zones on the purple formazan background. Ethanol 94% was sprayed on the plates to stop bacterial growth after the test.

6.7. Cytotoxicity tests

The development of multidrug-resistant tumor cells during the course of cancer chemotherapy is one of the most serious problems associated with the treatment. An important mechanism of acquiring the multidrug-resistance (MDR) phenotype in mammalian cells is the improved expression of a membrane glycoprotein (P-glycoprotein) (Gottesman and Pastan, 1993). Several active principles are known to surmount or circumvent MDR. Recently, some new tropane alkaloids isolated from two species of *Erythroxylum* were shown to be extremely potent inhibitors of MDR (Mi *et al.*, 2001; Mi *et al.*, 2002a). Similarly, the alkaloid extract and the isolated compounds (tropane alkaloid aromatic esters) of *E. vacciniifolium* were tested for their potential to reverse MDR. The activity was calculated as the amount of pure compound required to produce a response in 50% of the subjects to whom the compound was given (ED_{50}).

All these bioassay were carried out at the Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, Chicago (IL), USA under the direction of Prof. A. Douglas Kinghorn and Prof. John M. Pezzuto.

Cell Lines. Preliminary screening for the cytotoxicity activity of the tropane alkaloids was performed on human lung (Lu1), colon (Col2), oral epidermoid (KB) and hormone-dependant prostate (LNCaP) cancer cell lines. Also employed for preliminary testing were human telomerase reverse transcriptase-retinal pigment epithelial cells (hTERT-RPE1) and human umbilical vein endothelial cells (HUVEC). The hTERT-RPE1 was adopted as a substitute for a primary cell line for determining toxicity to normal human cells. The HUVEC line test system allowed identification of samples with potential antiangiogenic activity (Seo *et al.*, 2001). Assays involving carcinoma cell lines utilized established protocols (Likhitwitayawuid *et al.*, 1993), while HUVEC and hTERT-RPE1 cells were grown in more specialized media. HUVEC were purchased and grown in media and components supplied in the EGM-2 BulletKit (Clonetics Corporation, Walkersville, MD, USA) with 2% fetal bovine serum (FBS), while the hTERT-RPE1 line (Clotech Laboratories, Palo Alto , CA, USA) was maintained in DMEM/F-12 (Invitrotrogen Corporation, Carlsbad, CA, USA) containing 10% FBS. Both these cell lines were tested at an initial cell seed concentration of 5×10^4 cells/mL using standard assay procedures (Likhitwitayawuid *et al.*, 1993).

Multidrug-resistant human oral epidermoid carcinoma (KB-V1) cell lines were used for testing the inhibition of MDR in presence or absence of vinblastine (VLB; Sigma Chemical Co., St. Louis, MO, USA). KB-V1 cells were supplied by Dr I.B. Roninson (Department of Molecular Genetics, University of Illinois at Chicago, Chicago, IL, USA) and were grown in the

same medium as KB cells, which was further supplemented with VLB (1 μg/mL). Cell culture media and supplements were purchased from Life Technologies, Inc. (Grand Island, NY, USA).

Cytotoxic potential. The cytotoxic potential of test compounds with the various cell lines was determined as described in literature (Likhitwitayawuid *et al.*, 1993). In summary, different concentrations of test substances (dissolved in 10 μL of 10% DMSO) were transferred to 96-well plates, and 190 μL aliquots of cell suspensions (5×10^4 cells/mL) were added to each well. The plates were then incubated for 72 hours at 37°C (100% humidity with a 5% CO_2 atmosphere in air), and 100 μL of cold 20% aqueous trichloroacetic acid were added to the growth medium to fix the cells. The cultures were incubated for 30 min at 4°C, washed, air-dried, stained with sulforhodamine B solution, and washed with 1% acetic acid. After having added 200 μL of 10 mM Tris base to each well, the optical densities were measured at 515 nm using an enzyme-linked immunosorbent assay plate reader. A zero-day control was performed by adding an equivalent number of cells to several wells and incubating for 30 min at 37°C, and processing as described above. Optical density values obtained with zero-day control were subtracted, and cell survival, relative to control (solvent-treated) cultures, was calculated.

7. Quantitative determination of total alkaloids

The quantitative estimation of total alkaloids in the bark of *E. vacciniifolium* was calculated by two distinct methods, gravimetry and colorimetry using a UV-Vis spectrophotometer.

7.1. Gravimetric method

Stem bark powder (840.13 g) of authentic *E. vacciniifolium* was moistened with 20 mL of concentrated NH_4OH and exhaustively extracted with 3L of $CHCl_3$ three times during 24 hours at room temperature and under constant agitation. After filtration of the extracts, $CHCl_3$ fractions were combined and the solvent removed by rotary evaporation (Rotavapor Büchi) under vacuum at a maximum temperature of 35°C. Three accurately weighed parts of the lyophilized and homogenized extract (a: 335.6 mg; b: 330.2 mg; c: 335.7 mg) were transferred to separators with the aid of $CHCl_3$ (250 mL each) and extracted with successive portions (4 x 250 mL each) of a sulfuric acid solution (approximately 0.5 *N*). The layers were allowed to separate and the chloroform layers were discarded at the end of the extraction. The acid phases were combined and then made alkaline (pH 11) with some drops of concentrated ammonia. The precipitated bases were extracted four times with $CHCl_3$ (250 mL). The organic phases were filtered through 50 g of anhydrous sodium sulfate, previously washed with $CHCl_3$ and supported in a funnel with a small pledget of glass wool, into a weighed flask. The sodium sulfate was

washed and the combined organic phases evaporated under reduced pressure at a temperature below 35°C. The three extracts were lyophilized and precisely weighed.

7.2. Colorimetric method

The choice of a colorimetric procedure for the determination of total alkaloid content depends on the consideration that this method often gives more accurate results at low concentrations than the corresponding titrimetric or gravimetric procedures. Even though all constituents of the alkaloid extract seemed to have the same chromophore (a 1-methyl-1*H*-pyrrole-2-carboxylic acid esterifying the tropane nucleus) with a maximum of absorbance around 268 nm, the presence of other substances with absorption at this wavelength could interfere with the analysis. The eventual action of interfering substances was suppressed by the derivation of the tropane alkaloids and consequent shift of their maximum of absorbance at definite wavelength. In view of the selective character of the colorimetric reaction, it is important to control the operational procedure so that the color is specific for the components being determined. Alkaloids form with acidic indicators (*e.g.*, bromocresol green) a non-dissociable salt, which can be extracted with an organic solvent while the excess indicator anions remain in the water phase. The photometric intensity of the organic phase is then an indirect measure of the alkaloid concentration. Many reactions take place within well-defined limits of pH, so the specificity may be improved by adjusting the pH. The bromocresol green indicator used for this experiment has a transition interval from pH 4.0 to 5.4 with color changes from yellow to blue. After the reaction, the chloroform solution had a maximum absorbance at *ca*. 590 nm with pH 5.5.

In absorption spectrophotometry, the absorptivity of a substance is a constant independent of the intensity of the incident radiation, the internal cell length, and the concentration, with the result that concentration may be determined photometrically. A calibration curve was then constructed with different concentrations of a derived main alkaloid (catuabine D) and the quantitative determination of the constituents was carried out under the same experimental conditions.

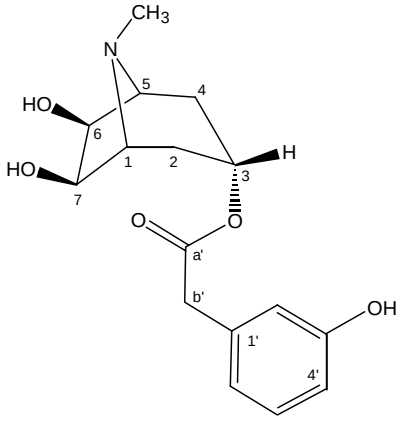
Assay preparation. An accurately weighted quantity of *E. vacciniifolium* bark powder (a: 0.9973 g; b: 0.9967 g; c: 0.9996 g) was mixed with 2.0 g of Kieselguhr (Fluka Chemie, Buchs, Switzerland) and moistened with 1.0 mL of a sodium carbonate solution (57% w/v). This mixture was placed in a percolator (15 cm x 1.5 cm i.d.) with approximately 30 mL of $CHCl_3$ and macerated overnight. The following day, the mixture was percolated slowly until the residue from a 10 mL portion of percolate, when dissolved in 0.5 mL of a DCM and MeOH mixture (3:1) and spotted on a TLC plate, was negative to Dragendorff reagent. The extraction was carried out during 6 hs and required 450 mL of $CHCl_3$. The organic solvent was evaporated

under vacuum to 200 mL, transferred to a 250-mL volumetric flask, and diluted to the required volume with $CHCl_3$. A portion of the organic solution (200.0 mL) was evaporated to dryness under reduced pressure and the residue was made up to 10.0 mL with pH 5.5 citrate buffer (9.054 g of citric acid and 40.796 g of sodium hydrogenphosphate were made up to 1000.0 mL with distilled water; the pH was adjusted with concentrated HCl). This solution was filtered though a paper filter and 5.0 mL of the filtrate were pipetted into a 60-mL separator. To the separator were added 5.0 mL of citrate buffer, 2.0 mL of a bromocresol solution (0.10 g of bromocresol green were dissolved in 2.4 mL of a 0.1 *N* sodium hydroxide solution and diluted to 100 mL with water), and finally 10 mL of $CHCl_3$. The mixture was shaken vigorously, the layers were allowed to separate, and the organic phase was taken. The extraction was repeated three more times with $CHCl_3$ (10 mL each) and finally the aqueous layer was discarded. The chloroform layers were combined and transferred to a 250.0-mL volumetric flask. Piperidine (3.0 mL) was added and the solution diluted to 250.0 mL with $CHCl_3$. The absorption of this solution were measured at 593 nm (maximal absorbance) after 15 minutes.

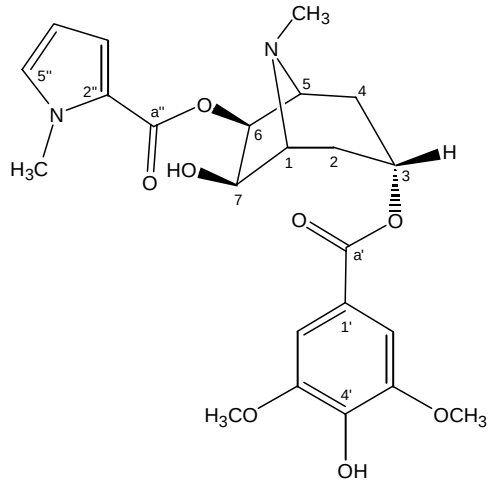
Standard curve. Standard solutions were prepared as follows: The pure compound catuabine D (10.3 mg) was dissolved with pH 5.5 citrate buffer into a 25-mL volumetric flask (*Solution A*). As directed under assay preparation, 5.0 mL of *Solution A* were transferred to a 60-mL separator, 5.0 mL of citrate buffer and 2.0 mL of bromocresol green were added, and the derived compound extracted four times with 10 mL of $CHCl_3$. The chloroform layers were combined and transferred to a 50.0-mL volumetric flask. Piperidine (3.0 mL) was added and the volumetric flask filled to the mark with $CHCl_3$ (*Solution B*). Seven dilutions were prepared from *Solution B* to establish a calibration curve in the range of $1.15\text{-}6.92 \cdot 10^{-5}$ M.

8. Physical constants and spectral data for the isolated compounds

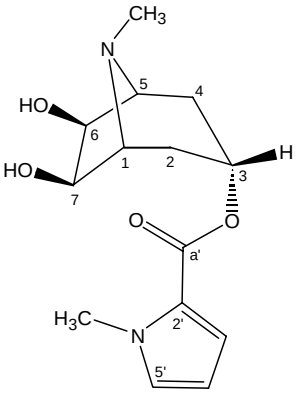
Compound **2**

Structure	
Chemical name	3α-(3-hydroxyphenylacetoxy)-6β,7β-dihydroxytropane
Trivial name	catuabine G
Formula	$C_{16}H_{21}NO_5$
Exact Mass	307.1420 Da
Molecular Weight	307.3417 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	+0.0 (*c* 0.57, EtOH)
UV λ_{max} (log ε)	217.7 (4.35), 276.3 nm (3.85, EtOH)
IR ν_{max} (KBr)	3455 (–OH), 2930 (C–H), 1735 (C=O), 690
^{1}H-NMR data	see Table 0-8
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	307 (10) $[M]^+$, 247 (15) $[M – OHCH=CHOH]^+$, 156 (10) $[M – 3\text{-}OHPhCH_2CO]^+$, 107 (20) $[3\text{-}OHPhCH_2]^+$, 95 (55) $[C_6H_9N]^+$, 94 (100) $[C_6H_8N]^+$
D/CI-MS *m/z*	308 $[M+H]^+$
HRESMS *m/z*	308.1493 $[M+H]^+$ (calculated for $C_{16}H_{22}NO_5$, 308.1492)

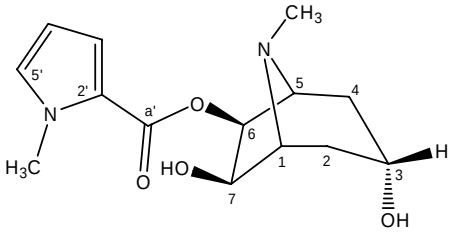
Compound **4**

Structure	
Chemical name	3α-(4-hydroxy-3,5-dimethoxybenzoyloxy)-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-7β-hydroxytropane
Trivial name	7β-hydroxycatuabine F
Formula	$C_{23}H_{28}N_2O_8$
Exact Mass	460.1846 Da
Molecular Weight	460.4771 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-2.6 (*c* 0.44, EtOH)
UV λ_{max} (log ε)	220.7 (4.16), 269.3 (4.13), 293.4 nm (3.71, EtOH)
IR ν_{max} (KBr)	3445 (–OH), 2945 (C–H), 1700 (C=O), 1415, 1330, 1220, 1110, 745
^{1}H-NMR data	see Table 0-8
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	460 (10) $[M]^+$, 335 (10) $[M - MpcOH]^+$, 263 (10) $[M - HdmbO]^+$, 181 (12) $[Hdmb]^+$, 138 (60) $[M - HdmbO - MpcOH]^+$, 122 (10) $[M - HdmbO - MpcO - OH]^+$, 108 (40) $[Mpc]^+$, 94 (100) $[C_6H_8N]^+$, 81 (15) $[C_5H_7N]^+$
D/CI-MS *m/z*	461 $[M+H]^+$
HRESMS *m/z*	461.1916 $[M+H]^+$ (calculated for $C_{23}H_{29}N_2O_8$, 461.1918)

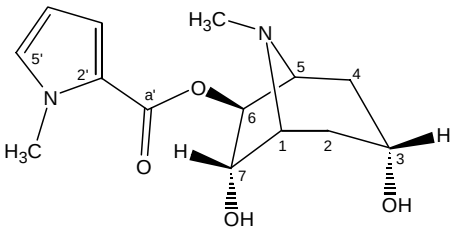
Compound **6**

Structure	
Chemical name	6β,7β-dihydroxy-3α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	7β-hydroxycatuabine I
Formula	$C_{14}H_{20}N_2O_4$
Exact Mass	280.1423 Da
Molecular Weight	280.3197 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-0.0 (*c* 0.60, EtOH)
UV λ_{max} (log ε)	267.4 nm (4.13, EtOH)
IR ν_{max} (KBr)	3500 (–OH), 2930 (C–H), 1705 (C=O), 1415, 1320, 1250, 1100, 745
^{1}H-NMR data	see Table 0-8
^{13}C-NMR data	see Table 0-9
EIMS *m/z* (rel. int.)	NA
D/CI-MS *m/z*	281 $[M+H]^+$
HRESMS *m/z*	281.1493 $[M+H]^+$ (calculated for $C_{14}H_{21}N_2O_4$, 281.1496)

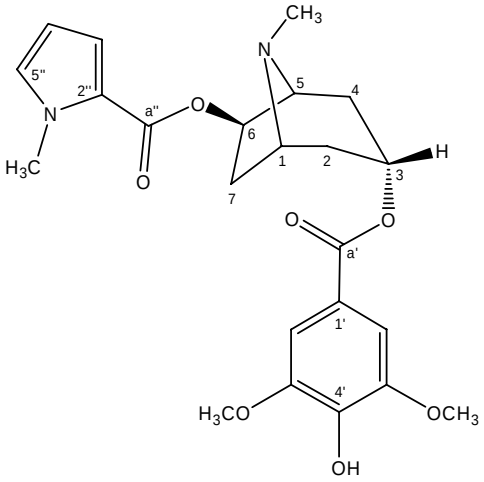
Compound **7**

Structure	
Chemical name	*3α*,7*β*-dihydroxy-6*β*-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	7*β*-hydroxycatuabine H
Formula	$C_{14}H_{20}N_2O_4$
Exact Mass	280.1423 Da
Molecular Weight	280.3197 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-1.5 (*c* 0.53, EtOH)
UV λ_{max} (log ε)	266.9 nm (4.04, EtOH)
IR ν_{max} (KBr)	3460 (–OH), 2930 (C–H), 1705 (C=O),1415, 1330, 1240, 1110, 750
^{1}H-NMR data	see Table 0-8
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	280 (10) $[M]^+$, 156 (25) $[M - MpcO]^+$, 155 (60) $[M - MpcOH]^+$, 138 (10) $[M - MpcOH - OH]^+$, 127 (35) $[M - Mpc - C_2H_5O]^+$, 113 (100) $[M - MpcO - C_2H_3O]^+$, 108 (80) $[Mpc]^+$, 96 (45) $[C_6H_{10}N]^+$, 94 (25) $[C_6H_8N]^+$, 82 (20) $[C_5H_8N]^+$
D/CI-MS *m/z*	281 $[M+H]^+$
HRESMS *m/z*	281.1492 $[M+H]^+$ (calculated for $C_{14}H_{21}N_2O_4$, 281.1496)

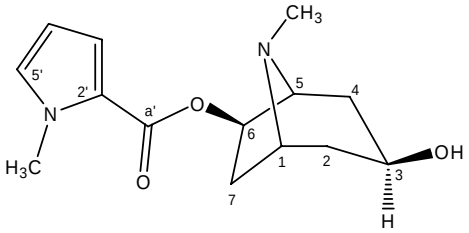
Compound **8**

Structure	
Chemical name	*3*α,7α-dihydroxy-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	7α-hydroxycatuabine H
Formula	$C_{14}H_{20}N_2O_4$
Exact Mass	280.1423 Da
Molecular Weight	280.3197 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-40.7 (*c* 0.57, EtOH)
UV λ_{max} (log ε)	268.1 nm (4.11, EtOH)
IR ν_{max} (KBr)	3390 (–OH), 2935 (C–H), 1700 (C=O),1415, 1320, 1250, 1110, 745
^{1}H-NMR data	see Table 0-8
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	280 (10) $[M]^+$, 156 (25) $[M – MpcO]^+$, 155 (45) $[M – MpcOH]^+$, 138 (10) $[M – MpcOH – OH]^+$, 127 (35) $[M – Mpc – C_2H_5O]^+$, 113 (100) $[M – MpcO – C_2H_3O]^+$, 108 (80) $[Mpc]^+$, 96 (55) $[C_6H_{10}N]^+$, 94 (30) $[C_6H_8N]^+$, 82 (20) $[C_5H_8N]^+$
D/CI-MS *m/z*	281 $[M+H]^+$
HRESMS *m/z*	281.1501 $[M+H]^+$ (calculated for $C_{14}H_{21}N_2O_4$, 281.1496)

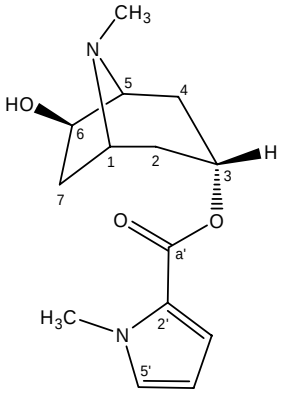
Compound **9**

Structure	
Chemical name	3α-(4-hydroxy-3,5-dimethoxybenzoyloxy)-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	catuabine F
Formula	$C_{23}H_{28}N_2O_7$
Exact Mass	444.1897 Da
Molecular Weight	444.4777 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-32.8 (*c* 0.46, EtOH)
UV λ_{max} (log ε)	220.7 (4.27), 270.0 (4.30), 293.4 nm (3.85, EtOH)
IR ν_{max} (KBr)	3420, 2945 (C–H), 1700 (C=O), 1415, 1330, 1215, 1110, 745
^{1}H-NMR data	see Table 0-8
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	444 (10) $[M]^+$, 247 (22) [M – HdmbO]$^+$, 181 (10) $[Hdmb]^+$, 138 (15) [M – Hdmb – MpcOH]$^+$, 122 (24) [M – HdmbO – MpcOH]$^+$, 108 (28) $[Mpc]^+$, 94 (100) $[C_6H_8N]^+$, 81 (15) $[C_5H_7N]^+$
D/CI-MS *m/z*	445 $[M+H]^+$
HRESMS *m/z*	445.1968 $[M+H]^+$ (calculated for $C_{23}H_{29}N_2O_7$, 445.1969)

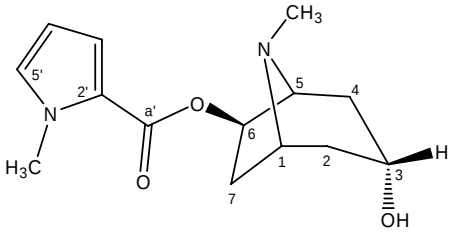
Compound **11**

Structure	
Chemical name	3β-hydroxy-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	isocatuabine H
Formula	$C_{14}H_{20}N_2O_3$
Exact Mass	264.1474 Da
Molecular Weight	264.3203 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-8.0 (*c* 0.41, EtOH)
UV λ_{max} (log ε)	266.6 nm (3.96, EtOH)
IR ν_{max} (KBr)	
^{1}H-NMR data	see Table 0-9
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	264 (20) $[M]^+$, 156 (20) $[M - Mpc]^+$, 139 (10) $[M - MpcOH]^+$, 122 (10) $[M - MpcOH - OH]^+$, 113 (100) $[M - Mpc - C_2H_3O]^+$, 108 (40) $[Mpc]^+$, 96 (40) $[C_6H_{10}N]^+$, 94 (35) $[C_6H_8N]^+$, 82 (10) $[C_5H_8N]^+$
D/CI-MS *m/z*	265 $[M+H]^+$
HRESMS *m/z*	265.1552 $[M+H]^+$ (calculated for $C_{14}H_{21}N_2O_3$, 265.1547)

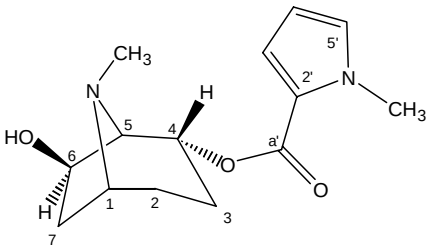
Compound **12**

Structure	
Chemical name	6β-hydroxy-3α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	catuabine I
Formula	$C_{14}H_{20}N_2O_3$
Exact Mass	264.1474 Da
Molecular Weight	264.3203 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-1.1 (*c* 0.39, EtOH)
UV λ_{max} (log ε)	267.1 nm (4.02, EtOH)
IR ν_{max} (KBr)	
^{1}H-NMR data	see Table 0-9
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	264 (10) $[M]^+$, 140 (25) $[M - MpcO]^+$, 139 (15) $[M - MpcOH]^+$, 108 (15) $[Mpc]^+$, 96 (15) $[C_6H_{10}N]^+$, 95 (60) $[C_6H_9N]^+$, 94 (100) $[C_6H_8N]^+$, 82 (10) $[C_5H_8N]^+$
D/CI-MS *m/z*	265 $[M+H]^+$
HRESMS *m/z*	265.1546 $[M+H]^+$ (calculated for $C_{14}H_{21}N_2O_3$, 265.1547)

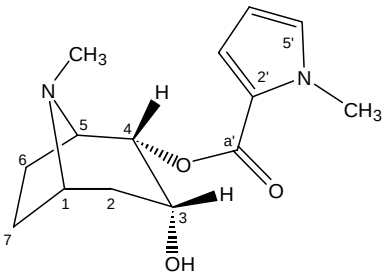
Compound **13**

Structure	
Chemical name	3α-hydroxy-6β-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	catuabine H
Formula	$C_{14}H_{20}N_2O_3$
Exact Mass	264.1474 Da
Molecular Weight	264.3203 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-9.6 (*c* 0.46, EtOH)
UV λ_{max} (log ε)	267.0 nm (4.10, EtOH)
IR ν_{max} (KBr)	
^{1}H-NMR data	see Table 0-9
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	264 (25) $[M]^+$, 113 (100) $[M - Mpc - C_2H_3O]^+$, 108 (30) $[Mpc]^+$, 96 (40) $[C_6H_{10}N]^+$, 94 (35) $[C_6H_8N]^+$, 82 (10) $[C_5H_8N]^+$
D/CI-MS *m/z*	265 $[M+H]^+$
HRESMS *m/z*	265.1546 $[M+H]^+$ (calculated for $C_{14}H_{21}N_2O_3$, 265.1547)

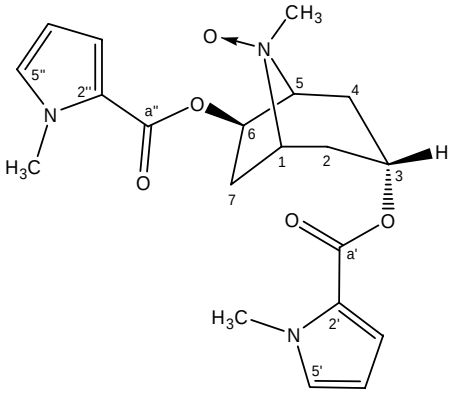
Compound **14**

Structure	
Chemical name	6α-hydroxy-4α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	vaccinine B
Formula	$C_{14}H_{20}N_2O_3$
Exact Mass	264.1474 Da
Molecular Weight	264.3203 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-2.0 (*c* 0.47, EtOH)
UV λ_{max} (log ε)	266.9 nm (4.10, EtOH)
IR ν_{max} (KBr)	3140 (–OH), 2950 (C–H), 1690 (C=O), 1415, 1330, 1245, 1120, 745
^{1}H-NMR data	see Table 0-9
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	264 (55) $[M]^+$, 156 (50) $[M - Mpc]^+$, 140 (20) $[M - MpcO]^+$, 139 (10) $[M - MpcOH]^+$, 108 (80) $[Mpc]^+$, 96 (30) $[C_6H_{10}N]^+$, 94 (10) $[C_6H_8N]^+$, 82 (100) $[C_5H_8N]^+$
D/CI-MS *m/z*	265 $[M+H]^+$
HRESMS *m/z*	265.1549 $[M+H]^+$ (calculated for $C_{14}H_{21}N_2O_3$, 265.1547)

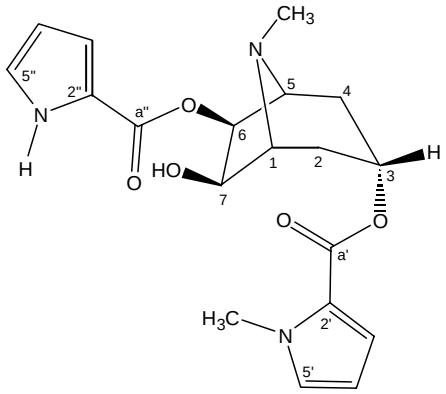
Compound **15**

Structure	
Chemical name	3α-hydroxy-4α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	vaccinine A
Formula	$C_{14}H_{20}N_2O_3$
Exact Mass	264.1474 Da
Molecular Weight	264.3203 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	+37.1 (*c* 0.51, EtOH)
UV λ_{max} (log ε)	268.1 nm (4.19, EtOH)
IR ν_{max} (KBr)	3390 (–OH), 2940 (C–H), 1695 (C=O), 1415, 1320, 1245, 1110, 740
^{1}H-NMR data	see Table 0-9
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	264 (25) $[M]^+$, 156 (20) $[M - Mpc]^+$, 140 (30) $[M - MpcO]^+$, 139 (20) $[M - MpcOH]^+$, 108 (70) $[Mpc]^+$, 96 (40) $[C_6H_{10}N]^+$, 94 (20) $[C_6H_8N]^+$, 82 (100) $[C_5H_8N]^+$
D/CI-MS *m/z*	265 $[M+H]^+$
HRESMS *m/z*	265.1547 $[M+H]^+$ (calculated for $C_{14}H_{21}N_2O_3$, 265.1547)

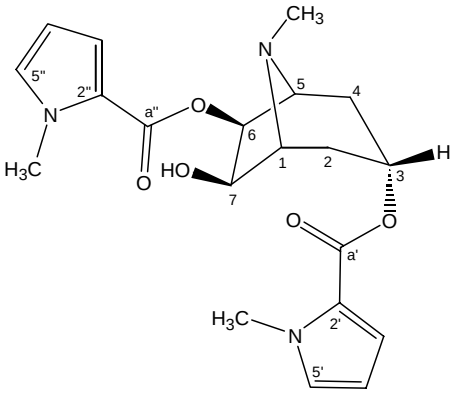
Compound **18**

Structure	
Chemical name	3α,6β-di-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane *N*-oxide
Trivial name	catuabine E *N*-oxide
Formula	$C_{20}H_{25}N_3O_5$
Exact Mass	387.1794 Da
Molecular Weight	387.4297 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-7.3 (*c* 0.33, EtOH)
UV λ_{max} (log ε)	268.2 nm (4.43, EtOH)
IR ν_{max} (KBr)	
^{1}H-NMR data	see Table 0-9
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	
D/CI-MS *m/z*	$[M+H]^+$
HRESMS *m/z*	$[M+H]^+$ (calculated for $C_{20}H_{26}N_2O_5$,)

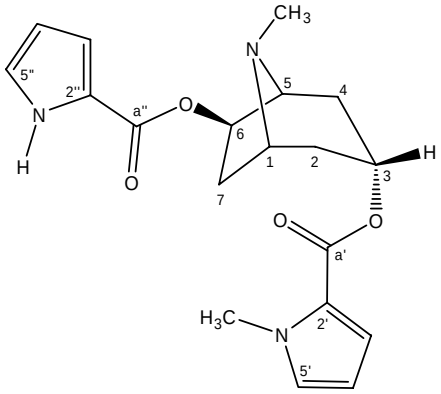
Compound **20**

Structure	
Chemical name	3α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-6β-[(1*H*-pyrrol-2-yl)carbonyloxy]-7β-hydroxytropane
Trivial name	7β-hydroxycatuabine D
Formula	$C_{19}H_{23}N_3O_5$
Exact Mass	373.1638 Da
Molecular Weight	373.4031 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-2.8 (*c* 0.40, EtOH)
UV λ_{max} (log ε)	267.2 nm (3.92, EtOH)
IR ν_{max} (KBr)	
^{1}H-NMR data	see Table 0-10
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	373 (10) $[M]^+$, 263 (7) $[M - PcO]^+$, 248 (10) $[M - MpcOH]^+$, 122 (10) $[M - PcO - MpcO - OH]^+$, 108 (45) $[Mpc]^+$, 95 (50) $[C_6H_9N]^+$, 94 (100) $[C_6H_8N]^+$, 81 (15) $[C_5H_7N]^+$
D/CI-MS *m/z*	374 $[M+H]^+$
HRESMS *m/z*	374.1709 $[M+H]^+$ (calculated for $C_{19}H_{24}N_3O_5$, 374.1710)

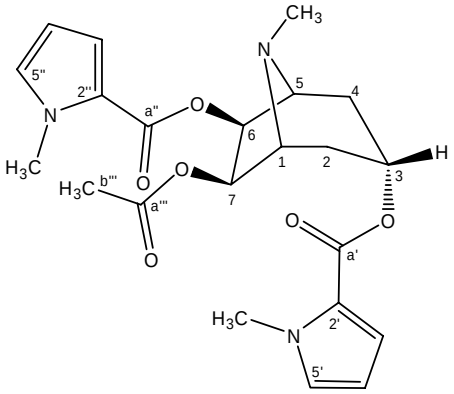
Compound **21**

Structure	
Chemical name	3α,6β-di-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-7β-hydroxytropane
Trivial name	7β-hydroxycatuabine E
Formula	$C_{20}H_{25}N_3O_5$
Exact Mass	387.1794 Da
Molecular Weight	387.4297 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	+0.8 (*c* 0.57, EtOH)
UV λ_{max} (log ε)	267.5 nm (4.41, EtOH)
IR ν_{max} (KBr)	3500 (–OH), 2945 (C–H), 1695 (C=O), 1410, 1320, 1250, 1115, 740
^{1}H-NMR data	see Table 0-10
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	387 (10) $[M]^+$, 262 (60) [M – MpcOH]$^+$, 154 (16) [M – Mpc – MpcOH]$^+$, 138 (80) [M – MpcO – MpcOH]$^+$, 137 (100) [M – MpcOH – MpcOH]$^+$, 122 (10) [M – MpcO – MpcO – OH]$^+$, 108 (100) [Mpc]$^+$, 94 (100) $[C_6H_8N]^+$, 81 (15) $[C_5H_7N]^+$
D/CI-MS *m/z*	388 $[M+H]^+$
HRESMS *m/z*	388.1869 $[M+H]^+$ (calculated for $C_{20}H_{26}N_3O_5$, 388.1867)

Compound **22**

Structure	
Chemical name	3α-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-6β-[(1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	catuabine D
Formula	$C_{19}H_{23}N_3O_4$
Exact Mass	357.1689 Da
Molecular Weight	357.4037 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-32.0 (*c* 0.58, EtOH)
UV λ_{max} (log ε)	266.8 nm (4.38, EtOH)
IR ν_{max} (KBr)	2935 (C–H), 1700 (C=O),1410, 1320, 1245, 1110, 740
^{1}H-NMR data	see Table 0-10
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	357 (55) $[M]^+$, 264 (18) $[M + H – Pc]^+$, 249 (15) $[M – Mpc]^+$, 233 (80) $[M – MpcO]^+$, 232 (75) $[M + H – MpcO]^+$, 140 (40) $[M + H – Pc – MpcO]^+$, 138 (32) $[M + H – Pc – MpcO]^+$, 122 (100) $[M – PcO – MpcOH]^+$, 108 (80) $[Mpc]^+$, 95 (100) $[C_6H_9N]^+$, 86 (95), 81 (35) $[C_5H_7N]^+$
D/CI-MS *m/z*	358 $[M+H]^+$
HRESMS *m/z*	358.1768 $[M+H]^+$ (calculated for $C_{19}H_{24}N_3O_4$, 358.17627)

Compound **23**

Structure	
Chemical name	3α,6β-di-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]-7β-acetoxytropane
Trivial name	7β-acetylcatuabine E
Formula	$C_{22}H_{27}N_3O_6$
Exact Mass	429.1900 Da
Molecular Weight	429.4664 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-36.8 (*c* 0.56, EtOH)
UV λ_{max} (log ε)	268.5 nm (4.45, EtOH)
IR ν_{max} (KBr)	2945 (C–H), 1745 (C=O), 1700 (C=O), 1410, 1320, 1250, 1110, 745
^{1}H-NMR data	see Table 0-10
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	429 (80) $[M]^+$, 321 (15) $[M - Mpc]^+$, 305 (100) $[M - MpcO]^+$, 245 (73) $[M - CH_3COOH - MpcO]^+$, 196 (20) $[M - Mpc - MpcOH]^+$, 180 (80) $[M - MpcO - MpcOH]^+$, 138 (32) $[M - CH_3COO - Mpc - MpcO]^+$, 122 (16) $[M - CH_3COO - MpcO - MpcO]^+$, 108 (100) $[Mpc]^+$, 94 (100) $[C_6H_8N]^+$, 81 (15) $[C_5H_7N]^+$
D/CI-MS *m/z*	430 $[M+H]^+$
HRESMS *m/z*	430.1967 $[M+H]^+$ (calculated for $C_{22}H_{28}N_3O_6$, 430.1973)

Compound **24**

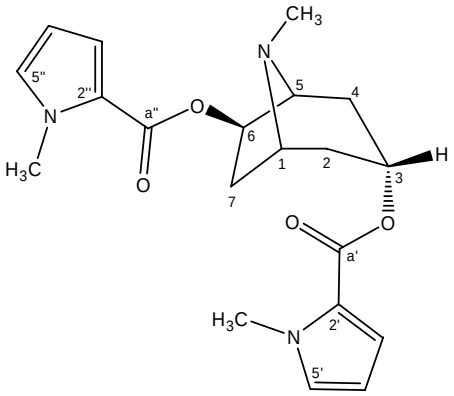
Structure	
Chemical name	3α,6β-di-[(1-methyl-1*H*-pyrrol-2-yl)carbonyloxy]tropane
Trivial name	catuabine E
Formula	$C_{20}H_{25}N_3O_4$
Exact Mass	371.1845 Da
Molecular Weight	371.4303 Da
Aspect	amorphous white solid
$[\alpha]_D^{20}$	-35.4 (*c* 0.57, EtOH)
UV λ_{max} (log ε)	267.6 nm (4.38, EtOH)
IR ν_{max} (KBr)	2945 (C–H), 1695 (C=O),1415, 1320, 1245, 1115, 740
^{1}H-NMR data	see Table 0-10
^{13}C-NMR data	see Table 0-11
EIMS *m/z* (rel. int.)	371 (30) $[M]^+$, 263 (5) $[M - Mpc]^+$, 247 (45) $[M - MpcO]^+$, 138 (15) [M – Mpc – MpcOH]$^+$, 122 (32) [M – MpcO – MpcOH]$^+$, 108 (55) $[Mpc]^+$, 95 (82) $[C_6H_9N]^+$, 94 (100) $[C_6H_8N]^+$, 86 (24), 81 (15) $[C_5H_7N]^+$
D/CI-MS *m/z*	372 $[M+H]^+$
HRESMS *m/z*	372.1924 $[M+H]^+$ (calculated for $C_{20}H_{26}N_3O_4$, 372.1918)

Table 0-7

Physical constants of the isolated compounds from *E. vacciniifolium*.

	$[\alpha]_D$ (EtOH)	UV λ_{max} (log ε) [nm] (EtOH)	IR ν_{max} [cm^{-2}] (KBr)	EIMS *m/z* (rel. int.)	D/CI-MS *m/z* $[M+H]^+$	HRESMS *m/z* $[M+H]^+$
2	+0.0 (*c* 0.57)	217.7 (4.35) 276.3 (3.85)	3455, 2930, 1735, 690	307 (10) $[M]^+$, 247 (15), 156 (10), 107 (20), 95 (55), 94 (100)	308	308.1493 (calcd for $C_{16}H_{22}NO_5$, 308.1492)
4	-2.6 (*c* 0.44)	220.7 (4.16) 269.3 (4.13) 293.4 (3.71)	3445, 2945, 1700, 1415, 1330, 1220, 1110, 745	460 (10) $[M]^+$, 335 (10), 263 (10), 181 (12), 138 (60), 122 (10), 108 (40), 94 (100), 81 (15)	461	461.1916 (calcd for $C_{23}H_{29}N_2O_8$, 461.1918)
6	-0.0 (*c* 0.60)	267.4 (4.13)	3500, 2930, 1705, 1415, 1320, 1250, 1100, 745		281	281.1492 (calcd for $C_{14}H_{21}N_2O_4$, 281.1496)
7	-1.5 (*c* 0.53)	266.9 (4.04)	3460, 2930, 1705, 1415, 1330, 1240, 1110, 750	280 (10) $[M]^+$, 156 (25), 155 (60), 138 (10), 127 (35), 113 (100), 108 (80), 96 (45), 94 (25), 82 (20)	281	281.1492 (calcd for $C_{14}H_{21}N_2O_4$, 281.1496)
8	-40.7 (*c* 0.57)	268.1 (4.11)	3390, 2935, 1700, 1415, 1320, 1250, 1110, 745	280 (10) $[M]^+$, 156 (25), 155 (45), 138 (10), 127 (35), 113 (100), 108 (80), 96 (55), 94 (30), 82 (20)	281	281.1501 (calcd for $C_{14}H_{21}N_2O_4$, 281.1496)
9	-32.8 (*c* 0.46)	220.7 (4.27) 270.0 (4.30) 293.4 (3.85)	3420, 2945, 1700, 1415, 1330, 1215, 1110, 745	444 (10) $[M]^+$, 247 (22), 181 (10), 138 (15), 122 (24), 108 (28), 94 (100), 81 (15)	445	445.1968 (calcd for $C_{23}H_{29}N_2O_7$, 445.1969)
11	-8.0 (*c* 0.41)	266.6 (3.96)		264 (20) $[M]^+$, 156 (20), 139 (10), 122 (10), 113 (100), 108 (40), 96 (40), 94 (35), 82 (10)	265	265.1552 (calcd for $C_{14}H_{21}N_2O_3$, 265.1547)
12	-1.1 (*c* 0.39)	267.1 (4.02)		264 (10) $[M]^+$, 140 (25), 139 (15), 108 (15), 96 (15), 95 (60), 94 (100), 82 (10)	265	265.1546 (calcd for $C_{14}H_{21}N_2O_3$, 265.1547)
13	-9.6 (*c* 0.46)	267.0 (4.10)		264 (25) $[M]^+$, 113 (100), 108 (30), 96 (40), 94 (35), 82 (10)	265	265.1546 (calcd for $C_{14}H_{21}N_2O_3$, 265.1547)
14	-2.0 (*c* 0.47)	266.9 (4.10)	3140, 2950, 1690, 1415, 1330, 1245, 1120, 745	264 (55) $[M]^+$, 156 (50), 140 (20), 139 (10), 108 (80), 96 (30), 94 (10), 82 (100)	265	265.1549 (calcd for $C_{14}H_{21}N_2O_3$, 265.1547)
15	+37.1 (*c* 0.51)	268.1 (4.19)	3390, 2940, 1695, 1415, 1320, 1245, 1110, 740	264 (25) $[M]^+$, 156 (20), 140 (30), 139 (20), 108 (70), 96 (40), 94 (20), 82 (100)	265	265.1547 (calcd for $C_{14}H_{21}N_2O_3$, 265.1547)
18	-7.3 (*c* 0.33)	268.2 (4.43)				(calcd for)
20	-2.8 (*c* 0.40)	267.2 (3.92)		373 (10) $[M]^+$, 263 (7), 248 (10), 122 (10), 108 (45), 95 (50), 94 (100), 81 (15)	374	374.1709 (calcd for $C_{19}H_{24}N_3O_5$, 374.1710)
21	+0.8 (*c* 0.57)	267.5 (4.41)	3500, 2945, 1695, 1410, 1320, 1250, 1115, 740	387 (10) $[M]^+$, 262 (60), 154 (16), 138 (80), 137 (100), 122 (10), 108 (100), 94 (100), 81 (15)	388	388.1869 (calcd for $C_{20}H_{26}N_3O_5$, 388.1867)
22	-32.0 (*c* 0.58)	266.8 (4.38)	2935, 1700,1410, 1320, 1245, 1110, 740	357 (55) $[M]^+$, 264 (18), 249 (15), 233 (80), 232 (75), 140 (40), 138 (32), 122 (100), 108 (80), 95 (100), 86 (95), 81 (35)	358	358.1768 (calcd for $C_{19}H_{24}N_3O_4$, 358.17627)
23	-36.8 (*c* 0.56)	268.5 (4.45)	2945, 1745, 1700, 1410, 1320, 1250, 1110, 745	429 (80) $[M]^+$, 321 (15), 305 (100), 245 (73), 196 (20), 180 (80), 138 (32), 122 (16), 108 (100), 94 (100), 81 (15)	430	430.1967 (calcd for $C_{22}H_{28}N_3O_6$, 430.1973)
24	-35.4 (*c* 0.57)	267.6 (4.38)	2945, 1695,1415, 1320, 1245, 1115, 740	371 (30) [M]+, 263 (5), 247 (45), 138 (15), 122 (32), 108 (55), 95 (82), 94 (100), 86 (24), 81 (15)	372	372.1924 (calcd for $C_{20}H_{26}N_3O_4$, 372.1918)

Table 0-8

^{1}HNMR data of alkaloids **2**, **4**, **6**-**9**.[a]

proton	**2**	**4**	**6**	**7**	**8**	**9**
1	3.02 br s	3.50 br s	3.21 br s	3.16 br s	3.35 m (6.4)	3.45 br s
2_{exo}	2.17 dd (15.6, 4.9)	2.55 m	2.32 m	2.20 m	2.17 dt (15.1, 4.9)	2.29 br dd (15.1, 4.9)
2_{endo}	1.52 d (15.6)	2.04 d (13.7)	1.76 d (15.6)	1.61 d (17.6)	1.89 d (15.1)	1.86 d (15.1)
3β	4.97 t (4.9)	5.36 t (4.9)	5.13 t (4.9)	4.11 t (4.9)	4.08 t (4.9)	5.31 t (4.9)
4_{exo}	2.17 dd (15.6, 4.9)	2.58 m	2.32 m	2.20 m	2.20 dt (15.1, 4.9)	2.32 br dd (15.6, 4.9)
4_{endo}	1.52 d (15.6)	2.07 d (15.1)	1.76 d (15.6)	1.64 d (17.6)	1.84 d (15.1)	2.07 d (15.6)
5	3.02 br s	3.72 br s	3.21 br s	3.27 br s	3.11 br s	3.40 br s
6α	4.18 s	5.91 d (6.4)	4.62 s	5.75 d (6.4)	5.42 d (2.4)	5.86 dd (7.3, 3.2)
7α	4.18 s	4.90 d (6.4)	4.62 s	4.84 d (6.4)		2.77 dd (14.2, 7.3)
7β					4.62 d (6.4)	2.30 m
N-CH_3	2.51 s	2.90 s	2.63 s	2.58 s	2.54 s	2.62 s
	3-OHPhCH_2CO	**Hdmb**	**Mpc**	**Mpc**	**Mpc**	**Hdmb**
2'	6.74 m	7.39 s	6.90 br s	7.02 dd (3.9, 2.0)	7.00 dd (3.9, 2.0)	7.39 s
3'			6.13 br s	6.10 dd (3.9, 2.4)	6.11 dd (3.9, 2.4)	
4'	6.76 m		6.88 br s	6.79 t (2.0)	6.81 t (2.0)	
5'	7.18 dd (8.8, 7.3)					
6'	6.76 m	7.39 s				7.39 s
N-CH_3			3.93 s	3.92 s	3.92 s	
b'	3.55 s					
O-CH_3		4.01 s				3.99 s
		Mpc				**Mpc**
3''		7.04 dd (3.9, 2.0)				6.91 dd (3.9, 2.0)
4''		6.13 dd (3.9, 2.0)				6.11 dd (3.9, 2.0)
5''		6.87 t (2.0)				6.81 t (2.0)
N-H						
N-CH_3		3.92 s				3.90 s

[a] Spectra recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard, δ values given in ppm, J values in parentheses given in Hz.

Table 0-9

^{1}HNMR data of alkaloids **11-15**, **18**.[a]

proton	**11**	**12**	**13**	**14**	**15**	**18**
1	3.60 br s	3.48 m (3.4)	3.35 m (3.9, 3.4)	3.33 br d (6.4)	3.26 br d	4.07 br s
2_{exo}	1.88 m	2.32 m	2.12 m (4.9)	1.88 m	1.86 m	2.53 m
2_{endo}	1.88 m	1.69 d (15.6)	1.66 d (14.7)	1.33 m	1.96 m	2.20 d (16.6)
3α	3.78 tt (10.8, 5.9)			1.36 m (6.4)		
3β		5.15 t (5.4)	4.08 t (4.9)	1.97 m	3.87 dd (8.8)	5.27 t (5.4)
4_{exo}	2.14 m	2.33 m	2.16 m 4.9	5.12 m (7.3, 3.4)	4.98 dd (8.8, 3.9)	2.51 m
4_{endo}	1.88 m	1.83 d (15.6)	1.91 d (14.7)			2.51 m
5	3.51 br s	3.23 br s	3.26 br s	3.18 d (3.4)	3.37 br d	4.07 br s
6α	5.24 dd (5.4)	4.72 dd (7.3, 2.9)	5.79 dd (7.3, 3.4)	4.55 dd (7.3, 3.4)	1.92 m	5.86 dd (8.3, 3.4)
6β					1.92 m	
7α	2.33 d (5.4)	2.75 dd (14.2, 7.3)	2.75 dd (13.7, 7.3)	2.09 m (7.3)	1.59 m	2.74 dd (13.2, 8.8)
7β	2.32 d (3.4)	2.08 dd (14.2, 7.3)	2.24 m	2.05 m (3.4)	2.08 m	3.19 m
N-CH_3	2.67 s	2.68 s	2.55 s	2.63 s	2.42 s	3.42 s
	Mpc	**Mpc**	**Mpc**	**Mpc**	**Mpc**	**Mpc**
3'	6.90 dd (3.9, 2.0)	6.91 dd (3.9, 2.0)	6.90 dd (3.9, 2.0)	6.95 dd (3.9, 2.0)	6.90 dd (3.9, 2.0)	7.07 dd (3.9, 1.5)
4'	6.11 dd (3.9, 2.4)	6.14 dd (3.9, 2.4)	6.09 dd (3.9, 2.4)	6.10 dd (3.9, 2.4)	6.11 dd (3.9, 2.4)	6.19 dd (3.9, 2.4)
5'	6.82 t (2.0)	6.83 dd (2.4, 2.0)	6.77 t (2.0)	6.78 dd (2.4, 2.0)	6.80 dd (3.9, 2.0)	6.86 t (2.0)
N-CH_3	3.92 s	3.93 s	3.93 s	3.92 s	3.91 s	3.96 s
						Mpc
3''						7.12 dd (3.9, 1.5)
4''						6.11 dd (3.9, 2.4)
5''						6.78 t (2.0)
N-CH_3						3.95 s

[a] Spectra recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard, δ values given in ppm, J values in parentheses given in Hz.

Table 0-10

^{1}HNMR data of alkaloids **20**-**24**.[a]

proton	**20**	**21**	**22**	**23**	**24**
1	3.56 br s	3.33 br s	3.40 br s	3.35 br s	3.50 br s
2_{exo}	2.43 m	2.27 br dd (17.1, 4.9)	2.22 br dd (15.1, 4.9)	2.28 br dd (15.1, 5.1)	2.34 m
2_{endo}	1.93 d (16.1)	1.72 d (17.1)	1.74 d (15.1)	1.87 d (15.1)	1.82 d (15.1)
3β	5.27 t (4.9)	5.24 t (4.9)	5.23 t (4.9)	5.28 t (5.1)	5.23 t (4.9)
4_{exo}	2.47 m	2.29 br dd (18.1, 4.9)	2.25 br dd (15.1, 4.9)	2.25 br dd (14.6, 5.1)	2.34 m
4_{endo}	1.96 d (15.6)	1.76 d (18.1)	1.96 d (15.1)	1.92 d (14.6)	2.06 d (15.6)
5	3.38 br s	3.20 br s	3.34 br s	3.35 br s	3.44 br s
6α	5.80 d (6.4)	5.71 d (5.9)	5.81 dd (7.3, 2.9)	5.88 d (6.3)	5.77 dd (7.6, 3.2)
7α	4.88 d (6.4)	4.79 d (5.9)	2.76 dd (13.9, 7.3)	5.81 d (6.3)	2.77 dd (14.4, 7.6)
7β			2.28 m		2.34 m
N-CH_3	2.78 s	2.61 s	2.58 s	2.65 s	2.63 s
	Mpc	**Mpc**	**Mpc**	**Mpc**	**Mpc**
3'	7.07 dd (3.9, 1.5)	7.13 dd (3.9, 2.0)	7.08 dd (3.9, 2.0)	7.22 dd (3.9, 2.0)	7.05 dd (3.9, 2.0)
4'	6.30 dd (3.9, 2.4)	6.18 dd (3.9, 2.0)	6.15 dd (3.9, 2.4)	6.18 dd (3.9, 2.0)	6.16 dd (3.9, 2.4)
5'	7.06 dd (2.4, 1.5)	6.81 t (2.0)	6.80 t (2.0)	6.80 t (2.0)	6.80 t (2.4, 2.0)
N-CH_3	3.95 s	3.93 s	3.94 s	3.92 s	3.93 s
	Pc	**Mpc**	**Pc**	**Mpc**	**Mpc**
3''	7.10 dd (3.9, 2.0)	7.03 dd (3.9, 2.0)	6.91 dd (3.9, 2.0)	6.93 dd (3.9, 2.0)	6.91 dd (3.9, 1.5)
4''	6.13 dd (3.9, 2.4)	6.11 dd (3.9, 2.0)	6.25 dd (3.9, 2.0)	6.09 dd (3.9, 2.0)	6.10 dd (3.9, 2.4)
5''	6.86 t (2.0)	6.79 t (2.0)	6.94 dd (3.9, 2.0)	6.78 t (2.0)	6.79 t (2.4, 2.0)
N-H			9.58 br s		
N-CH_3		3.94 s		3.93 s	3.94 s
				CH_3COO	
b'''				2.04 s	

[a] Spectra recorded in $CDCl_3$ at 499.87 MHz using TMS as internal standard, δ values given in ppm, *J* values in parentheses given in Hz.

Table 0-11

^{13}CNMR data of alkaloids **2**, **4**, **6-9**, **11-15**, **18**, **20-24**.[a]

carbon	**2**	**4**	**6**	**7**	**8**	**9**	**11**	**12**	**13**	**14**	**15**	**18**	**20**	**21**	**22**	**23**	**24**
1	65.5	68.0	66.1	66.8	62.0	60.3	61.4	59.3	60.4	59.8	60.5	72.5	64.6	63.7	65.5	65.4	60.5
2	27.2	30.0	28.0	30.9	28.4	34.8	37.4	31.2	37.7	25.7	38.15	34.5	29.3	28.1	33.7	31.4	35.0
3	66.9	65.8	65.3	64.0	62.7	67.0	62.4	65.3	64.1	23.8	67.3	61.2	65.5	65.9	65.9	65.6	65.5
4	27.2	30.1	28.0	31.1	32.5	33.5	35.9	29.6	36.8	67.3	77.3	33.4	29.3	28.2	32.2	31.2	33.4
5	65.5	65.0	66.1	64.1	64.0	65.8	67.3	67.6	66.6	70.9	63.1	76.6	67.4	66.5	59.6	64.8	66.4
6	73.3	75.9	73.5	77.7	84.6	78.0	76.2	74.8	79.2	72.6	22.5	74.2	76.5	77.6	79.1	76.5	78.2
7	73.3	74.3	73.5	75.8	78.9	36.2	35.6	39.2	35.6	39.8	26.7	35.0	74.6	75.9	36.4	77.7	35.6
N-CH_3	35.1	37.9	35.7	36.1	37.6	40.3	39.1	37.0	40.6	37.8	38.7	48.9	37.2	36.1	39.2	38.9	40.4
	3-OHPhCH_2CO	**Hdmb**	**Mpc**	**Mpc**	**Mpc**	**Hdmb**	**Mpc**	**Mpc**	**Mpc**	**Mpc**	**Mpc**	**Mpc**	**Mpc**	**Mpc**	**Mpc**	**Mpc**	**Mpc**
1'	134.5	119.5				120.7											
2'	115.6	106.5	121.7	122.1	122.0	106.6	121.7	122.2	122.8	122.5	122.0	121.6	121.8	122.3	122.5	122.2	122.4
3'	156.6	147.2	117.3	118.3	118.6	147.0	118.2	117.6	117.7	118.0	118.3	118.5	118.5	118.2	117.8	118.5	117.8
4'	113.8	140.3	107.6	107.9	107.9	139.7	108.0	108.0	107.7	107.8	107.9	108.6	110.1	108.3	108.2	108.5	108.2
5'	129.2	147.2	129.8	129.8	129.9	147.0	130.2	130.0	129.4	129.6	129.9	130.6	124.0	129.9	129.7	129.9	129.8
6'	119.9	106.5				106.6											
N-CH_3			36.0	36.8	36.7		36.7	36.6	36.7	36.8	36.8	36.8	36.3	36.7	36.8	36.7	36.7
a'	170.4	165.4	159.9	161.0	161.6	165.6	160.5	160.2	161.2	160.4	161.3	159.6	160.2	160.2	160.3	160.1	160.2
b'	41.4																
O-CH_3		55.9				56.3											
		Mpc				**Mpc**						**Mpc**	**Pc**	**Mpc**	**Pc**	**Mpc**	**Mpc**
2''		121.2				122.2						122.5	121.4	122.0	123.0	122.1	122.4
3''		118.1				117.8						119.3	116.0	118.3	115.3	117.9	117.9
4''		107.7				107.8						108.1	107.9	107.9	110.3	107.8	107.9
5''		129.9				129.7						129.7	130.0	129.8	122.9	129.6	129.7
N-CH_3		36.0				36.5						37.1		36.7		36.7	36.7
a''		160.0				160.6						161.3	160.7	160.8	160.9	160.3	160.8
																CH_3COOH	
a'''																170.2	
b'''																20.8	

[a] Spectra recorded in $CDCl_3$ at 125.70 MHz using $CDCl_3$ as internal standard, δ values given in ppm. The ^{13}C assignments were ascertained by DEPT, HSQC and HMBC experiments.

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Abstract

Throughout the ages, men and women have incessantly pursued every means to increase, preserve or recapture their sexual capacity, or to stimulate the sexual desire of selected individuals. One of the most recurrent methods has been the use of natural aphrodisiacs.

Nowadays, the commercialization of new synthetic "love drugs", *e.g.* Viagra®, Cialis® and Levitra®, has fascinated the public interest and has led to a reassessment of classical aphrodisiacs and to the search for new ones. The practice of self-medication by an increasing number of patients, the incessant aggressive advertising of these herbal aphrodisiacs, the invasion of the medicinal market with uncontrolled dietary supplements and the absence of real directives amplifies the potential health hazards to the community.

In order to evaluate the possible risks of commercialized aphrodisiac products on consumer health, the development and validation of a rapid qualitative and quantitative method for the analysis of yohimbine in these products, is reported in the first part of the present work. Yohimbine, a pharmacologically well-characterized α_2-adrenoceptor antagonist with activity in the central and peripheral nervous system, has been used for over a century in the treatment of erectile dysfunction. The analytical method is based on liquid chromatography coupled with ultraviolet and mass spectrometry (LC-UV-MS) and in total, 20 commercially-available aphrodisiac preparations were analyzed. The amount of yohimbine measured and expressed as the maximal dose per day suggested on product labels ranged from 1.32 to 23.16 mg.

The second part of this work involved the phytochemical and pharmacological investigation of *Erythroxylum vacciniifolium* Mart. (Erythroxylaceae), a plant used in Brazilian traditional medicine as an aphrodisiac and tonic, and locally known as *catuaba*. With the aim of obtaining preliminary structure information on-line, the alkaloid extract was analyzed by high performance liquid chromatography (HPLC) coupled to diode array UV detection (LC-UV-DAD), to mass spectrometry (LC-MS) and to nuclear magnetic resonance spectroscopy (LC-NMR). Interpretation of on-line spectroscopic data led to structure elucidation and partial identification of 24 potentially original alkaloids bearing the same tropane skeleton.

Seventeen new tropane alkaloids were then isolated from the alkaloid extract of the plant, including catuabines D to I, their derivatives and vaccinines A and B. All compounds were elucidated as tropane-diol or -triol alkaloids esterified by at least one 1-methyl-1*H*-pyrrole-2-carboxylic acid. One of the isolated compounds was identified as a tropane alkaloid *N*-oxide. Their structures were determined by high resolution mass spectrometry and multi-dimensional NMR spectroscopy.

Among the numerous bioassays undertaken, only the cytotoxicity tests exhibited a weak positive activity of certain compounds.

Résumé

De tout temps, hommes et femmes ont cherché par tous les moyens à développer, préserver ou recouvrer leurs propres capacités sexuelles mais également à stimuler le désir du partenaire. L'utilisation d'aphrodisiaques naturels a été l'un des recours les plus répandus. De nos jours, la commercialisation de nouvelles "love drugs" de synthèse, *e.g.* Viagra®, Cialis®, Levitra®, a remis au goût du jour les aphrodisiaques classiques et à relancer la recherche sur des molécules nouvelles. La pratique croissante de l'automédication, le matraquage publicitaire sur les aphrodisiaques naturels, la prolifération sur le marché de compléments alimentaires non contrôlés et l'absence de véritable législation accroissent les risques qui pèsent sur la santé publique.

Dans le but d'évaluer les risques potentiels sur le consommateur de produits aphrodisiaques commercialisés, le développement et la validation d'une méthode rapide d'analyse qualitative et quantitative de la yohimbine dans ces préparations du marché sont exposés dans la première partie de ce travail. La yohimbine est un antagoniste α_2-adrénocepteur du système nerveux central et périphérique, elle est employée depuis plus d'un siècle dans le traitement des dysfonctionnements érectiles. Cette méthode analytique utilise la chromatographie liquide couplée à l'ultraviolet et à la spectrométrie de masse (LC-UV-MS) et au total, vingt préparations aphrodisiaques ont été étudiées. La dose journalière de yohimbine mesurée s'est révélée très variable selon les produits puisqu'elle varie de 1.32 à 23.16 mg.

La seconde partie de ce travail concerne l'étude phytochimique et pharmacologique d'*Erythroxylum vacciniifolium* Mart. (Erythroxylaceae), une plante, appelée localement *catuaba*, utilisée dans la médecine traditionnelle brésilienne comme tonique et aphrodisiaque. Dans un premier temps, l'extrait alcaloïdique a été analysé par chromatographie liquide haute performance (HPLC) couplée soit à un détecteur UV à barrette d'iode (LC-UV-DAD), soit à un spectromètre de masse (LC-MS), ou soit à un spectromètre de résonance magnétique nucléaire (LC-RMN). L'interprétation de ces données spectrales enregistrées en ligne a permis d'obtenir des informations structurales et d'identifier partiellement près de 24 alcaloïdes appartenant à la classe des tropanes et potentiellement originaux.

Par des méthodes classiques de chromatographie liquide sur l'extrait alcaloïdique de la plante, dix sept tropanes nouveaux ont ensuite été isolés dont les catuabines et leurs dérivés, et les vaccinines. Tous ces composés sont des tropane-diols ou triols estérifiés par au moins un groupe acide 1-méthyl-1*H*-pyrrole-2-carboxylique. Un de ces composés a été identifié comme un tropane *N*-oxyde. Toutes les structures ont été déterminées par spectrométrie de masse haute résolution et spectroscopie RMN multi-dimensionnelle.

Parmi les nombreux tests biologiques réalisés sur ces tropanes, seuls les tests de cytotoxicité se sont révélés faiblement positifs pour certains de ces composés.