ANALYTICAL STUDIES OF SOME AGENTS FOR FERTILITY REGULATION

A Thesis presented by

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MALE FERTILITY REGULATION

Dedication

To Chris,

for all his help and support

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Summary

This Thesis describes three pieces of work, all of which were commissioned by the World Health Organization Special Programme in Human Reproduction which are concerned with aspects of the chemistry of agents for the regulation of fertility. The first two topics are related to agents causing infertility in the male, whilst the third is related to a method of contraceptive delivery in the female.

Gossypol is a chiral natural product with phenolic and aldehyde functionality, occurring in the cotton plant. The literature on the isolation, identification, chemistry and biological properties of gossypol is reviewed in this Thesis. Following reports by Chinese scientists that racemic gossypol causes reversible infertility in male animals and human beings, it became clear from further studies in both China and in Western countries that toxic side effects were seen on prolonged dosing. In an attempt to lower the toxicity of this interesting antispermatogenic agent, attention turned to studies of the individual enantiomers, which were first separated in Matlin's group by derivatisation with a chiral amine and chromatography of the resulting diastereomeric Schiff's bases on a non-chiral (normal or reverse) phase. Following hydrolysis and fractional crystallization, 40 g of chemically and optically pure (-)-gossypol was prepared and micronized for a toxicology study.

Extracts of Tripterygium wilfordii L. Hook have been used in Traditional Chinese Medicine for many centuries for the treatment of inflammatory and dermatological disorders. Recent studies in China have revealed that men undergoing prolonged treatment with tabletted extracts of T. wilfordii

suffered a reversible loss of fertility and tests in male rats have shown that this is related to a dramatic reduction in sperm density and motility. This Thesis gives a literature review of all the known constituents of T. wilfordii and the biological studies which have been reported. Furthermore, it describes a detailed bioassay-directed sub-fractionation of both T. wilfordii tablets and of crude extracts of the plant, obtained from China. This work led to the isolation and identification of a series of diterpene epoxides, including the known triptolide and tripdiolide and some isomers of the latter, one of which is a new compound, which show varying degrees of activity as oral anti-spermatogenic agents in male rats. Extensive use was made of analytical and preparative HPLC and of spectroscopic techniques, including high field NMR and mass spectrometry, in accomplishing the identification of the antifertility constituents of T. wilfordii.

In the final part of the Thesis, a literature review is presented of the design, mode of action and clinical studies of vaginal rings. These are slow-release drug delivery systems in which a contraceptive steroid is contained within a polymer ring and is released by diffusion processes at a slow rate for prolonged periods of time. The Thesis then describes experimental studies in which suitable, constant eluent conditions and analytical procedures were developed and successfully applied. Release rates were measured for two types of newly fabricated rings, designed to release either progesterone or levonorgestrel at a near constant rate for periods of three months. For both steroids, it was possible to show that release could be described mathematically by a simple diffusion-distance model and that reproducible in

vitro release rates could be obtained for devices in which a fixed loading of steroid is dispersed within a polymer core surrounded by an outer polymer sheath. Suitable dimensions were selected for the production of rings providing an initial release rate of 5 mg/day of progesterone and quality control was carried out on four batches of these rings fabricated by Dow Corning Ltd for WHO clinical trials. Similarly, suitable ring dimensions were selected for the production of rings providing an initial release rate of 20 ug/day of levonorgestrel and the production of these rings for clinical use in Britain and other countries is now being undertaken by Roussel Laboratories.

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PART B: FEMALE FERTILITY REGULATION

PART A: MALE FERTILITY REGULATION

CHAPTER 1

MALE REPRODUCTION

1.1 Introduction

The testes fulfil a dual role: an endocrine function in the formation of steroid hormones (androgens) and a germinal one in the production of spermatozoa. Within the testes are the seminiferous tubules, which occupy 85% of the gland volume. They are highly coiled and are embedded in connective tissue containing the Leydig cells, the site of steroidogenesis. In contrast to the seminiferous tubules, the Leydig cells have an excellent blood and lymphatic supply. The insides of the coiled seminiferous tubules are lined with Sertoli cells, which nourish and maintain the germ cells as they develop into spermatozoa.

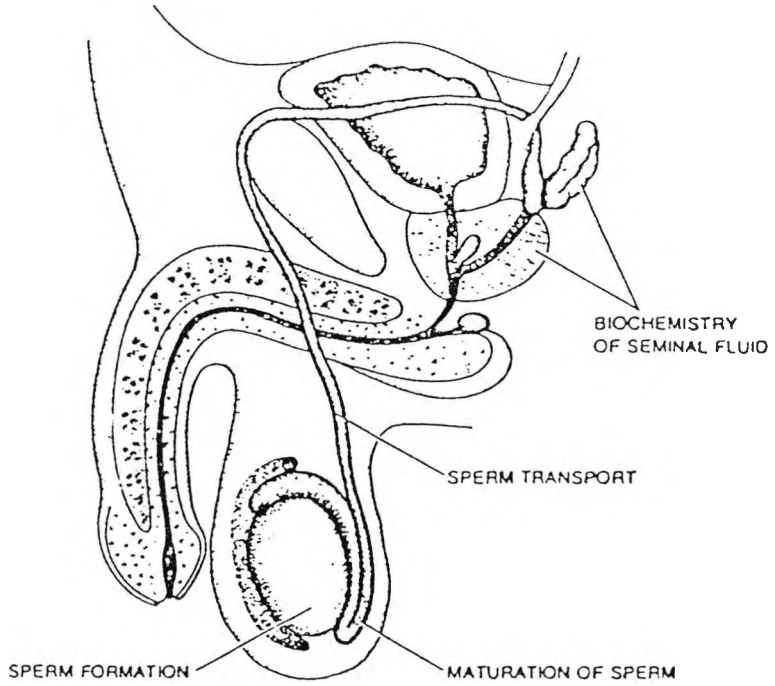


Fig. 1-1: Male reproductive organs

The testes differ from the ovaries in that there are no marked cyclic changes in the production of the steroid hormones. The same two hormones controlling the ovary, FSH and LH, also control the testis. They are secreted by the male pituitary gland under the control of the releasing hormone (Gn-RH or LH-RH) from the hypothalamus. The effect of FSH in the male is to stimulate the Sertoli cells which participate in spermatogenesis and to promote the growth of the seminiferous tubules, while LH stimulates the growth and steroid hormone production of the Leydig cells. One of the main products of the Leydig cells is testosterone, which is required by the Sertoli cells for maintaining spermatogenesis. Testosterone is involved in feedback control and modulates the release of LH by depressing hypothalamic output of Gn-RH (Fig. 1-3). In addition to their role in spermatogenesis, testosterone and related androgens such as 5α-dihydrotestosterone and androstenedione have important anabolic and androgenic effects on bone, muscle and hair growth and maintain the prostate, seminal vesicles and other accessory organs. They also play an important role in the mating behaviour of the male, a sufficient serum level of androgen being essential to maintain libido and potency.

Neither Sertoli cells nor germ cells possess LH binding activity. The Sertoli cell's response to LH involves increased secretory activity including the biosynthesis of a unique androgen-binding protein (ABP). Sertoli cells contain receptors for FSH, which also stimulates ABP formation and in addition that of a peptide called inhibin. Inhibin is probably responsible for the selective feedback control of FSH from the anterior pituitary gland. The general function of the Sertoli cells is to provide a supportive environment

for the developing germ cells and making ABP which is responsible for creating a localized high concentration of testosterone within the seminiferous tubules.

The gonadal steroids move through the bloodstream and/or extracellular fluids to reproductive organ target cells, where they bind to receptors that carry them into the nucleus. There they activate or depress the genes in the DNA which make new proteins and thus affect the organ's structure or function (Fig. 1-2).

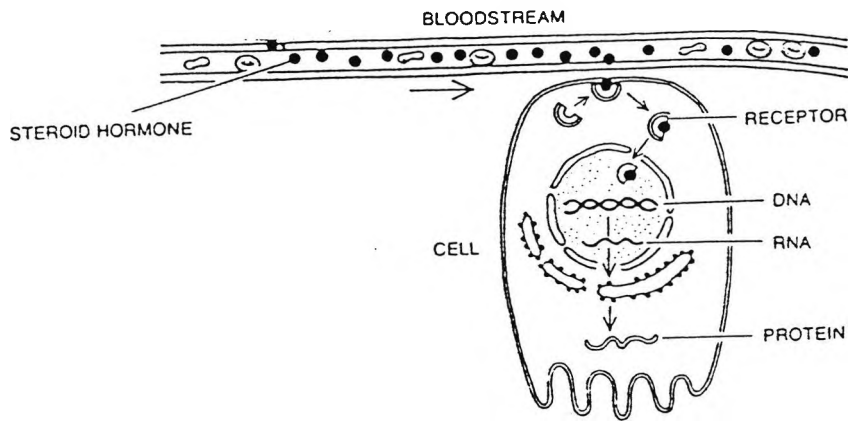


Fig. 1-2 Transport of steroids to reproductive organ target cell.

In contrast to the explosive increase of the oogonia in the embryonic ovary, the stem cells or spermatogonia in the embryonic testis do not proliferate significantly, until full spermatogenesis begins at puberty.

Development (Fig. 1.3) starts with the mitotic division of spermatogonia (SG) lying on the inner surface of the basement membrane of the seminiferous tubules, and proceeds, through successive mitotic divisions, with production of various intermediate forms of spermatogonia to the next morphologically distinct type, the spermatocyte (SC). A proportion of spermatogonia remain as such in a resting state preventing depletion of stem cells for later spermatogenesis.

The spermatocytes undergo further meiotic divisions and adhere to the Sertoli cells to form spermatids (ST), which progressively ripen into mature spermatozoa, reaching the stage when they are released from the Sertoli cell into the lumen of the seminiferous tubules. These stages are known as spermatogenesis (Fig. 1-3). At this point the spermatozoan is metabolically inactive and incapable of active movement.

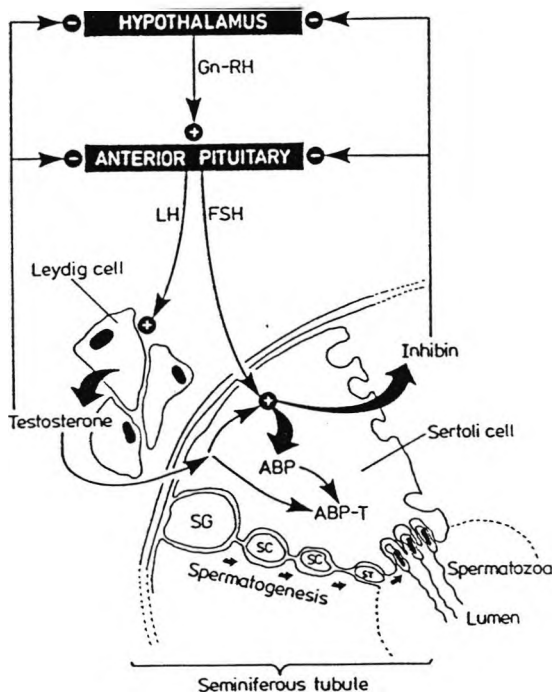


Fig. 1-3: Interaction of gonadotrophins with spermatogenesis

The spermatozoa leave the lumen of the seminiferous tubules into the rete testis and then into the epididymis, which is a highly convoluted tube of 7 metres length in man. The sperm is passively transported through the epididymis where it undergoes its final maturation process taking approximately 24 days. At the end of this period the spermatozoa are motile and capable of fertilization, and waiting to be ejaculated. Spermatogenesis is a continuous process taking about 74 days in man.

During ejaculation the accessory glands, such as the prostate and the seminal vesicles, add further components to seminal fluid after its passage through the vas deferens (Fig. 1-1). The most significant additions, in terms of quantity, are citric acid and fructose.

The spermatozoan (Fig. 1-4) is divided into head, middle piece and tail. In the centre of the head is the nucleus, and over the anterior surface of the nucleus spreads the acrosome, forming a cap. The acrosome is necessary for penetration of the sperm into the oocyte. The mitochondria, packed in a spiral round the axis of the flagellum, form the midpiece, and they create the energy to give motility to the tail of the sperm.

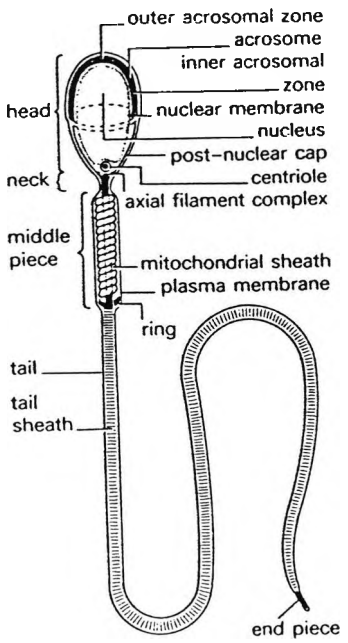


Fig. 1-4: Diagram of spermatozoan

1.2 Male contraception

Physiologically there are more sites where reproduction can be interrupted in male than in female, and there are also more profound biological problems. Men produce new sperm throughout their adult lives at a rate of more than 10^8 per day and there is no natural mechanism for turning off fertility in the way that pregnancy prevents further ovulation in women through the suppressive effect of progesterone.

Male contraception consist generally of inhibition of spermatogenesis or prevention of fertilization. The problem in male contraception is the fact that the individual drugs must not influence testicular testosterone production and secondary male characteristics. Furthermore, the substances should not have mutagenic effects because by mutation of one spematogonium the genetic defect will be reproduced many times.

Substances blocking spermatogenesis and leading to infertility should fulfil the following conditions :

1. They should selectively block spermatogenesis without influencing the hypophyseal control of the testis (via gonadotrophin secretion and inhibiting testicular testosterone production).
2. They should have no influence on spermatogonia and spermatocytes, which would cause an irreversible loss of fertility and possible genetic mutations.

Fertility in the male can be inhibited by substances which block spermatogenesis or fertilization ability. Gonadal steroids can be used for male contraception, because of their inhibition of pituitary gonadotrophins, which leads to suppresion of spermatogenesis with subsequent azoospermia.

Since the discovery of the gonadotrophin releasing hormone (GnRH), which is a decapeptide (Fig. 1-5), many analoges has been formed by small changes in the natural GnRH (agonists) or by more substantial changes leading to the production of antagonists.

The pituitary Gn-RH receptor is normally stimulated in a pulsatile manner by Gn-RH secreted by the hypothalamus. Continuous stimulation by exogenous natural Gn-RH or it's synthetic analogues eventually desensitises the receptors and renders them refractory to further stimulation. The consequent down-regulation of pituitary function and failure of FSH and LH secretion will lead to a severe decline in spermatogenesis. Similarly, the antagonists, which block the Gn-RH receptors without stimulating a pituitary response, will also cut off secretion of FSH and LH. Both approaches have been shown to lead to very low levels of sperm production, although total azoospermia appears to be very difficult to achieve. A major obstacle to the use of these Gn-RH analogues for male contraception is that the down-regulation of LH results in a fall in levels of circulating androgens and consequent loss of libido and potency. Indeed, both types of analogues are clinically used in the treatment of prostatic cancer, which is androgen -dependent, as an alternative to castration. For use in male contraception, the depleted blood androgen levels must be restored by administration of exogenous androgens, e.g. testosterone esters. This requires a careful balancing act, since secondary sexual characteristics must be maintained without re-stimulating spermatogenesis at the testicular level. Further, practical difficulties arise because the peptide Gn-RH analogues can not be orally administered and

must be given as injections of slow-release microcapsules. Moreover, at the present time there are no satisfactory orally-absorbable androgens and these too must be given as long-acting injectable formulations.

It has been found that aminoacids at both ends of the Gn-RH's peptide chains are essential for both receptor binding and biological activity. During long-term application of Gn-RH analogues it has been found necessary to supplement with testosterone simultaneously to prevent a decrease in serum testosterone and changes in the secondary male characteristics, including libido. By the combination of Gn-RH analogues and testosterone in one treatment, spermatogenesis could be supressed whilst androgen deficiency could be prevented.

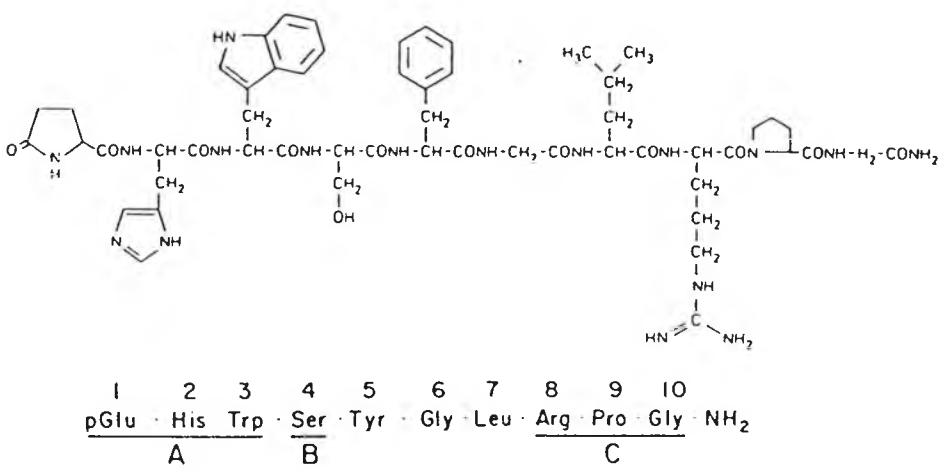


Fig. 1-5: Structure of gonadotrophin releasing hormone

Down-regulation of the hypothalamic-pituitary-gonadal axis can also be achieved by using the negative feedback effects of steroids to switch off Gn-RH production.

Large doses of androgens can be used, but as noted above must be given by injection and they tend to have undesirable

side effects (anabolic effects, acne) whilst incompletely shutting down spermatogenesis. Progestins have also been tried, again with partial success and with added complications such as feminisation and gynaecomastia. Estrogens also have potential use but would pose unacceptable risks of cardiovascular disease at the high doses necessary. Again, both progestins and estrogens have the further disadvantage of simultaneously down-regulating both FSH and LH production and require the simultaneous administration of a counterbalancing androgen to maintain libido and potency.

The ideal approach, at the pituitary level, would be an agent which selectively suppressed only FSH production, whilst leaving the LH/testosterone system undisturbed. As noted earlier, the gonadal peptide inhibin is believed to be a natural feedback regulator of FSH and it has been speculated that it could be used as a male contraceptive. Inhibin has been identified as a complex, high molecular weight glycoprotein and is currently being produced by genetic molecular engineering methods. To date, there have not been sufficient quantiti es available to test its contraceptive potential. If it proves to be effective, the cost of production and the method of administration will be serious problems to be resolved before it can be used clinically.

Another approach to male contraception is the search for non-steroidal compounds which can interfere directly with spermatogenesis without blocking the production of testosterone, thus avoiding interference with secondary male characteristics. In the present work, two projects have been undertaken for the World Health Organization concerning the chemistry of non-steroidal anti-spermatogenic agents.

1.2.1 Large scale resolution of gossypol

The observation that gossypol, a polyphenolic pigment contained in the raw cotton seed, gave rise to unintentional temporary male infertility in some regions of China with only minor apparent side effects, prompted the worldwide study of this phenomenon. In extensive clinical trials in China in the 1970's, amongst the most serious side effects were a very low incidence of hypokalemia and the more unacceptable and more frequent incidence of irreversible sterility after prolonged administration. It was later discovered that the active antifertility compound was the (-)-enantiomeric form of gossypol. It was hoped that use of pure (-)-gossypol would lead to antifertility action with a lower dose of drug, and that toxicity would be significantly reduced. The development and application of large scale methods for resolution of (±)-gossypol are described in Chapter 3.

1.2.2 Antifertility components of Tripterygium wilfordii

Investigation of male Chinese arthritis sufferers treated with a traditional Chinese herbal medicine from Tripterygium wilfordii roots indicated the antifertility effect of this drug. The main observation was the sharp decline of motility and density of the epididymal spermatozoa of treated subjects, without any apparent effect on the endocrine system. The reversibility of fertility suppresion after cessation of treatment was promising.

This project is concerned with the isolation and structure determination of the active compounds in Tripterygium wilfordii. Activity was established by combining fractionation procedures with a bioassay involving examination of sperm quality and fertility in dosed male rats. Results are presented in Chapter 5.

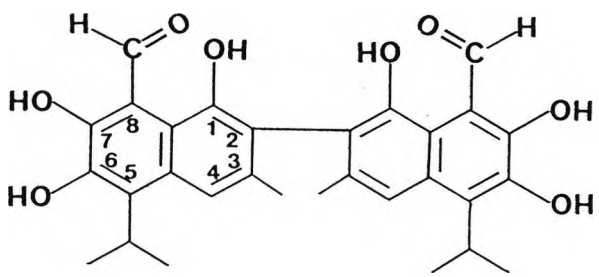
CHAPTER 2

GOSSYPOL: A REVIEW OF CHEMICAL AND BIOLOGICAL ASPECTS

2.1 Introduction

Gossypol (1) is a polyphenolic yellow pigment which has been known for nearly a century as a toxic contaminant of the cotton plant. It has a molecular weight of 518.54 ($C_{30}H_{30}O_8$).

Interest in the pharmacology of gossypol was aroused when it was shown that people consuming cotton seed oil in China became infertile. Chinese scientists discovered that oral doses of racemic gossypol acetic acid induced infertility in men by interfering with spermatogenesis. Thus the possibility existed for the development of a "male pill". Furthermore, within the last decade it has been found that gossypol is a cytotoxic agent against a wide variety of tumour cells and it is currently being investigated as a potential clinical anti-cancer agent.



(1)

Gossypol

2.2 Gossypol in cotton plant: content, localization and isolation procedures

The polyphenolic gossypol pigments are indigenous in the genus Gossypium. In the cotton plant they are contained almost exclusively within discrete bodies called pigment glands, which are found in leaves, stems, roots and seeds of cotton plants.

0.5-3.0% gossypol is found in the bark of cotton plant roots, insignificant amounts are present in leaves, stalk, boll valves, seed hulls and pericarp. More significant amounts are found in the flowers. However, the greatest gossypol concentration is found in the seeds, specifically in the kernels in an amount which varies from 0.002 to 6.64%.[1]

Gossypol was first isolated by Longmore[2] from cotton seed oil in 1886, though it proved to be very impure. In 1899 Marchlewski[3] isolated gossypol from the same source and refined it by treating an ether solution of technical gossypol with acetic acid, thus precipitating gossypol acetic acid (a 1:1 molecular complex). Marchlewski ascertained the polyphenolic structure of the compound and therefore called it "gossypium phenol"-gossypol.

Carruth[4] also obtained pure gossypol from an ether extract from cottonseed oil. Campbell et al[5], Kozhevnikova and Gil'tburg[6,7], and Rzhekhin[8] worked with defatted crushed cotton seeds and kernels. Boatner[9] treated an ether extract of pigment glands ruptured after wetting while Royce[10] and coworkers treated an ether extraction of ground root bark. Another isolation method is based on the weak acidic properties of the phenolic gossypol.[4] The ether extract of cotton seeds is treated with dilute caustic soda solution,

which extracts the gossypol sodium salt into the aqueous phase. After addition of ether and acidification, free gossypol is extracted into the ether layer. Gossypol is then purified by precipitation as gossypol acetic acid.

A further method of isolation is by treating the ether extract containing gossypol with aniline, (Carruth[4], King and Thurber[11]), giving the dianilinogossypol Schiff's base (4). Free gossypol is formed by treating dianilinogossypol with alcoholic sodium hydroxide and steam distillation to remove the aniline. The gossypol is then purified via the gossypol acetic acid complex.

In all the methods of isolation described, sodium dithionite is present to prevent oxidation of gossypol, and only peroxide free ether is used.

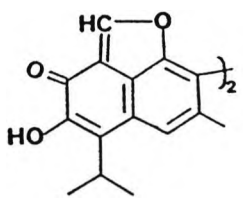
2.3 Early structural studies

Although Marchlewski[3] in 1899 had detected free hydroxyl groups in the gossypol molecule, he failed to produce crystalline acetyl and benzoyl derivatives. In 1918 Carruth[4] was also unsuccessful, but he obtained 3 gossypol derivatives, which proved to be very important in the elucidation of the structure of gossypol:

-Anhydrogossypol (2): the product obtained by splitting off 2 water molecules on heating gossypol.

-Apogossypol (3): the product obtained by treating gossypol with strong alkali, resulting in the loss of two carbonyl groups.

-Dianilinogossypol (4): the condensation product of 1 gossypol molecule with 2 of aniline, accompanied by the loss of 2 water molecules.



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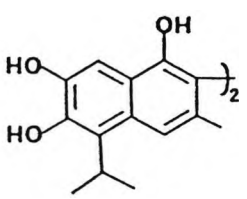

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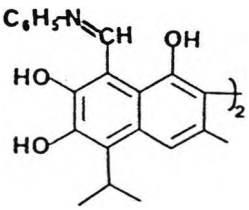


(4)

In 1928 Clark[12] succeeded in determining the molecular formula of gossypol, $C_{30}H_{30}O_8$. Clark proved the presence of 6 hydroxyl groups in the gossypol molecule by obtaining an hexacetate on acetylation. By treating gossypol with hydroxylamine he obtained the dioxime, indicating the presence of 2 carbonyl groups. Therefore Clark proposed this partial structural formula for gossypol $C_{28}H_{24}(CO)_2(OH)_6$.

Amongst the decomposition products after strong oxidation of gossypol with potassium permanganate, Clark detected formic, acetic and isobutyric acids, indicating the presence of branched aliphatic side chains.[12]

Adams, Geissman, Kirkpatrick and coworkers[13-21] elucidated the structure of gossypol in a series of papers published in 1937-1941. A large number of reactions of gossypol, its derivatives and their degradation products were studied.

The absorption spectra of gossypol and its derivatives were indicative of a molecule containing two naphthalene nuclei connected either directly or through an alkane chain. This fact was supported by the study of the degradation products of gossypol and its derivatives.

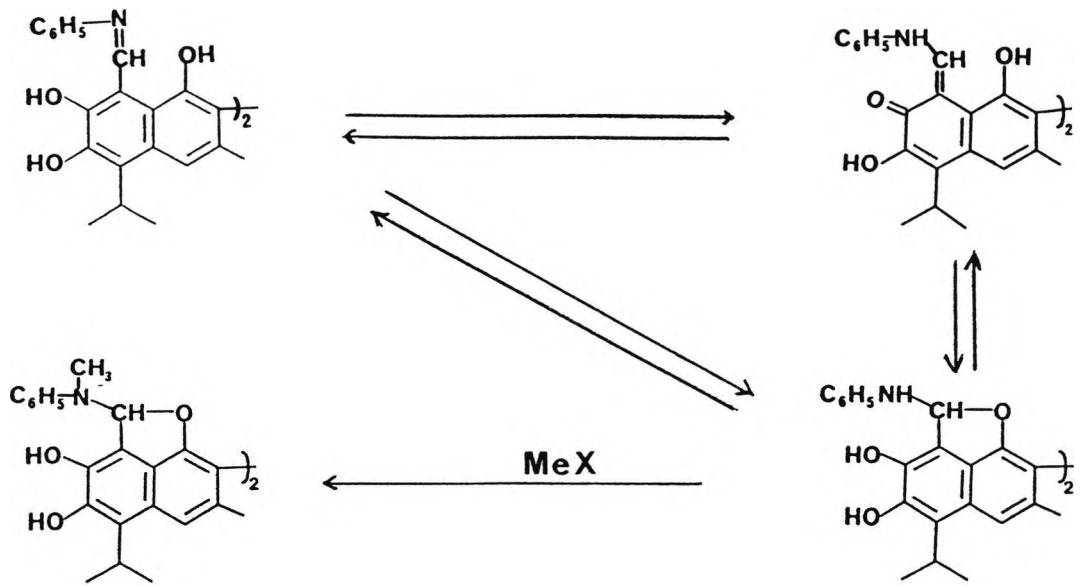
Working on characteristic colour reactions and salts from the naphthalene series, the following conclusions could be drawn:

1. Gossypol has two phenolic hydroxyl groups ortho to each other, as proved by the formation of a green colour when reacted with ferric chloride.[22]
2. Gossypol has a hydroxyl group peri or ortho to a carbonyl group, as indicated through the deep red colour obtained on reaction with pyroboroacetate[23] and a purple red colour when reacted with stannic chloride.[24] Gossypol titrates as a dibasic acid and forms a dipyridyl salt, characteristic of hydroxyaldehydes.

The existence of two carbonyl groups in the molecule of gossypol was confirmed by the condensation product formed between 1 mol of gossypol and 2 mols of aniline or ammonia derivatives with the elimination of two mols of water. All these condensation products are readily hydrolysed by acid to gossypol.

Of importance from the structural point of view were the methylation and acetylation of the yellow dianilinogossypol. Under proper conditions a red dimethylated compound is

isolated which has the methyl groups attached to nitrogens, which cannot be removed on hydrolysis by hydriodic acid. This experiment establishes the probability of the dianilino compound existing not as a simple Schiff's base but as an equilibrium between three tautomeric forms of an o-hydroxyimine (Scheme 2-1).



Tautomeric forms of dianilinogossypol

Scheme 2-1.

Adams and coworkers[13-21] did further work on methylated and acetylated derivatives of gossypol, as well as on anhydrogossypol (2) to prove beyond doubt the existence of the gossypol in three tautomeric forms.

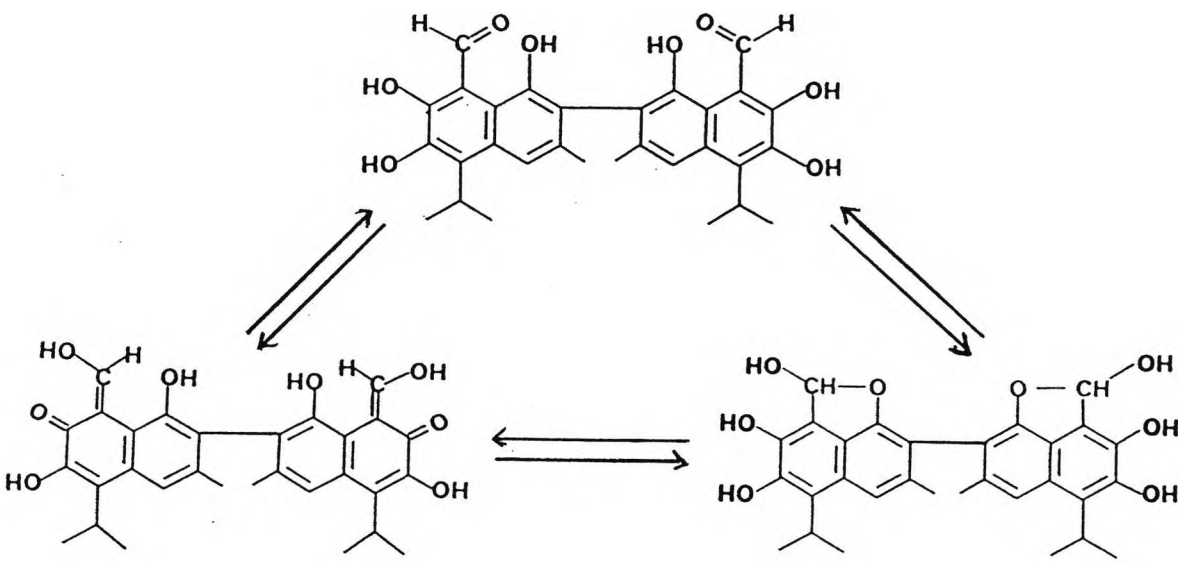
A white tetramethyl ether of gossypol (5) was obtained by the action of dimethyl sulphate in the presence of alkali. Further methylation of the compound gave a white or red hexamethyl ether (6), depending on the experimental conditions.

Gossypol hexaacetate was formed similarly by reacting gossypol with acetic anhydride and sodium acetate under very mild conditions.

The non reactivity of gossypol tetra- and hexamethyl ethers and gossypol hexaacetate in neutral or alkaline solution with many carbonyl reagents, such as condensation with amines or oxidation of the aldehyde group, led to closer study of the aldehyde group structure.

In acid solution however, the derivatives of gossypol condensed in a ratio 1:2 with primary amines and were also attacked by other characteristic carbonyl reagents.

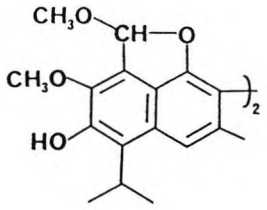
Consequently Adams and coworker postulated the structure of gossypol represented by the various tautomeric forms (Scheme 2-2).



Tautomeric forms of gossypol

Scheme 2-2

It was therefore believed that in the tetra- and hexaalkyl ethers of gossypol, the system involving the two aldehyde groups and two of the six hydroxyls may be stabilized in the lactol form (compounds 5 and 6) by alkylation with the formation of two acetal linkages.



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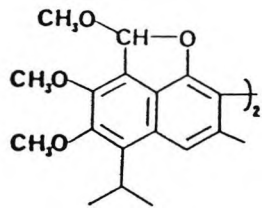
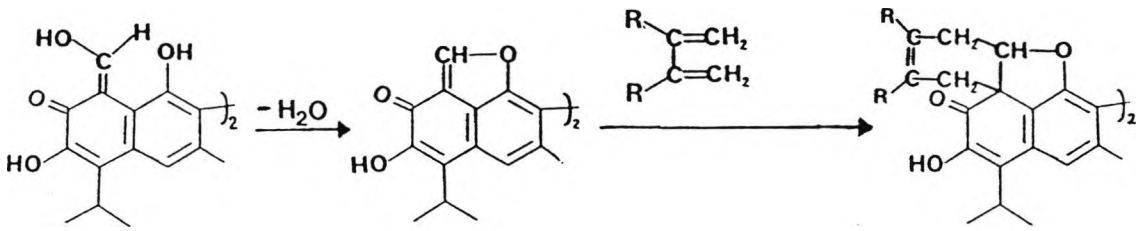


(6)

The study of anhydrogossypol (2) gave further evidence of the tautomeric structure of gossypol. Anhydrogossypol is produced by heating gossypol (7) above its melting point or by the use of a mild dehydrating agent in an inert solvent. Anhydrogossypol is very sensitive to hydrolysis, regenerating gossypol. Its reactions with nucleophiles always involve simple addition while similar derivatives are obtained with the loss of H_2O in the case of gossypol. A very easily opened oxygen containing ring must therefore be present.

Anhydrogossypol (2) reacts with both butadiene and dimethylbutadiene to give stable addition products (8: R = H or Me). This indicates the probability of an electron-deficient double bond in the oxygen-containing ring.



(7) (2) (8)

Scheme 2-3.

Gossypol also adds dienes but with the simultaneous loss of water to give the same compound as those obtained from anhydrogossypol (Scheme 2-3).

The anhydrogossypol diene addition product (8) also contains two residual hydroxy groups which are readily methylated or acetylated.

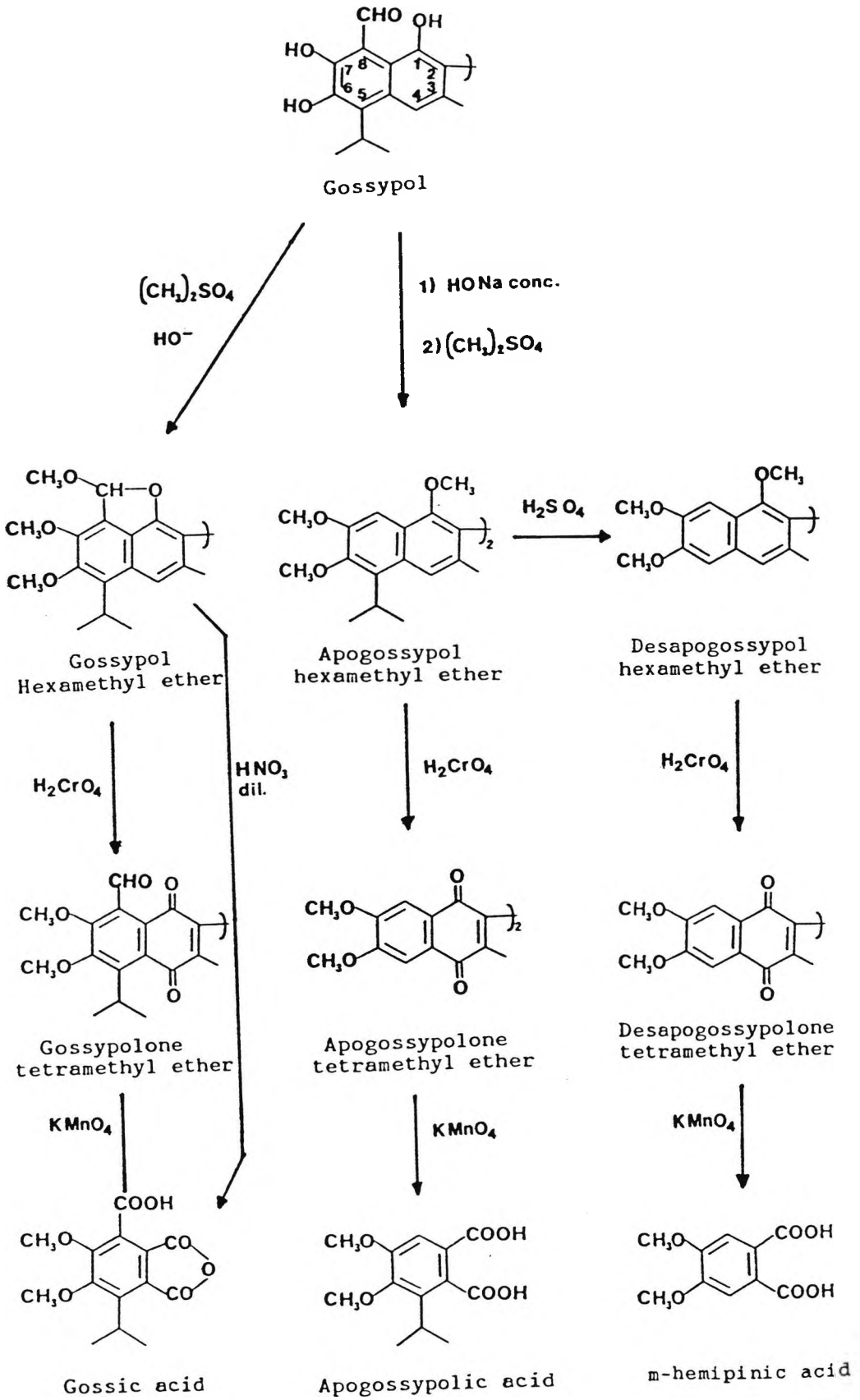
Further information about the structure of gossypol came from study of the oxidation products of gossypol hexamethyl ether (6) and the hexamethyl ethers of gossypol derivatives, such as apogossypol (3) and desapogossypol (Scheme 2-4).

Apogossypol (3) is formed by the loss of two carbons and two oxygens when gossypol is reacted with concentrated aqueous sodium hydroxide. The unstable apogossypol is formed by the loss of the two aldehyde groups, a reaction common to many hydroxyaldehydes of the benzene and naphthalene series. The hexamethyl ether of apogossypol, however, is a stable compound.

Desapogossypol hexamethyl ether is formed by the loss of C_6H_{12} when apogossypol hexamethyl ether reacts with cold concentrated sulphuric acid. This product still contains 6 methoxyl groups (Scheme 2-4).

Gossypol-, apogossypol- and desapogossypol-hexamethyl ethers are individually oxidized with chromic acid to the respective quinones gossypolone-, apogossypolone-, and desapogossypolone tetramethyl ether (Scheme 2-4).

Gossypolone, apogossypolone and desapogossypolone oxidize under the action of potassium permanganate to water soluble products called gossic acid, apogossypolic acid and hemipinic acid (Scheme 2-4). Apogossypolic acid and hemipinic acid are both ortho dicarboxylic benzoic derivatives, where the only difference between their molecular formulas is a C_3H_6 residue.


Gossypol
(CH3)2SO4
HO⁻
1) HONa conc.
2) (CH3)2SO4
Gossypol Hexamethyl ether
Apogossypol hexamethyl ether
H2SO4
Desapogossypol hexamethyl ether
H2CrO4
HNO3 dil.
H2CrO4
H2CrO4
Gossypolone tetramethyl ether
Apogossypolone tetramethyl ether
Desapogossypolone tetramethyl ether
KMnO4
KMnO4
KMnO4
Gossic acid
Apogossypolic acid
m-hemipinic acid

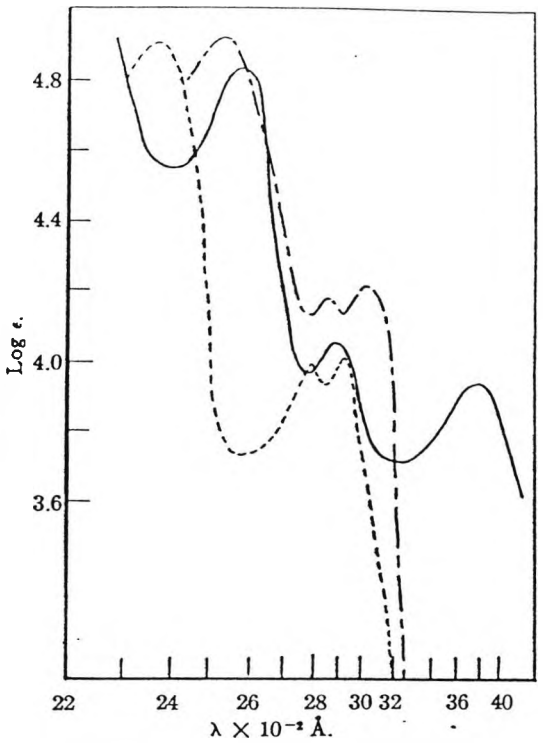
Scheme 2-4

By distilling the dilute sulphuric acid filtrate from the preparation of desapogossypol, a distillate is obtained which on oxidation gives a test for acetone. This demonstrates that the C_3H_6 groups are isopropyls which are being removed directly from the naphthalene nuclei by hydrolysis. The isopropyl group can only be located in position 5. Gossic acid is an anhydride of an ortho dicarboxylic benzene derivative.

The experimental facts presented by Adams and coworkers established the functional groups in the 1, 5, 6, 7 and 8 positions of two naphthalene nuclei. There was also certainty of hydrogen in the 4 position.

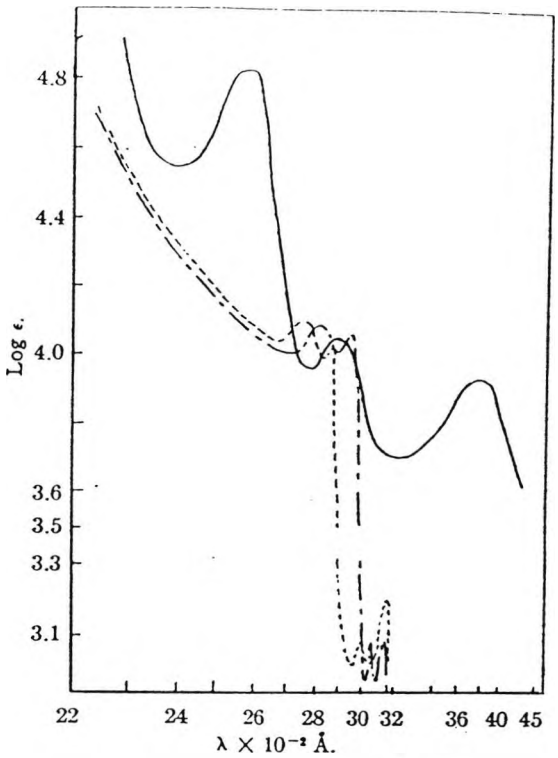
The remaining points needing to be established were the position linkage of the naphthalene nuclei and the location of the methyl group.

An examination of the absorption spectra of gossypol and many of its derivatives shows them all to be characterized by an absorption maximum at approximately 250 nm. with the log $_E$ close to 5 and a maximum near 300 nm, with a log $_E$ of 4. A comparison of the absorption spectra of gossypol with α-α and β-β binaphthalene (Figure 2-1) and also with sym-di-α and sym-di-β-naphthylethane (Figure 2-2) indicates that gossypol contains a binaphthalene chromophore. The enhancement of the intensity in the gossypol spectrum compared to naphthalene derivatives is an amount which could be satisfactorily explained by assuming double the number of naphthalene rings per mole in gossypol.



(1)—, Gossypol; (2)—-, β,β-binaphthyl; (3)---, α,α-binaphthyl.

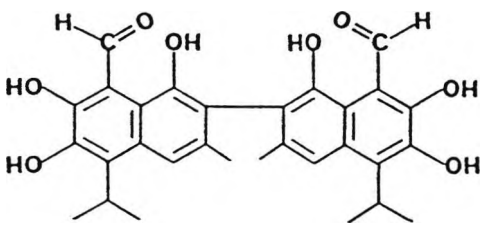
Figure 2-1



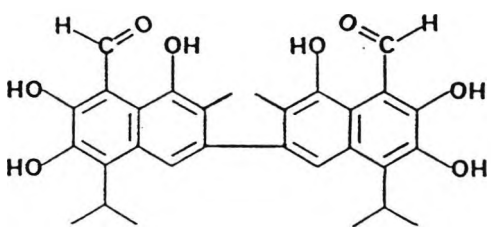
(1) —, Gossypol; (2) ---, *sym*-di-β-naphthylethane; (3) —-, *sym*-di-α-naphthylethane.

Figure 2-2

Adams and coworker arrived at two possible structures of gossypol depending on the location of the methyl group and the joining of the naphthalene nuclei in position 2 or 3 (compounds 9 and 10).



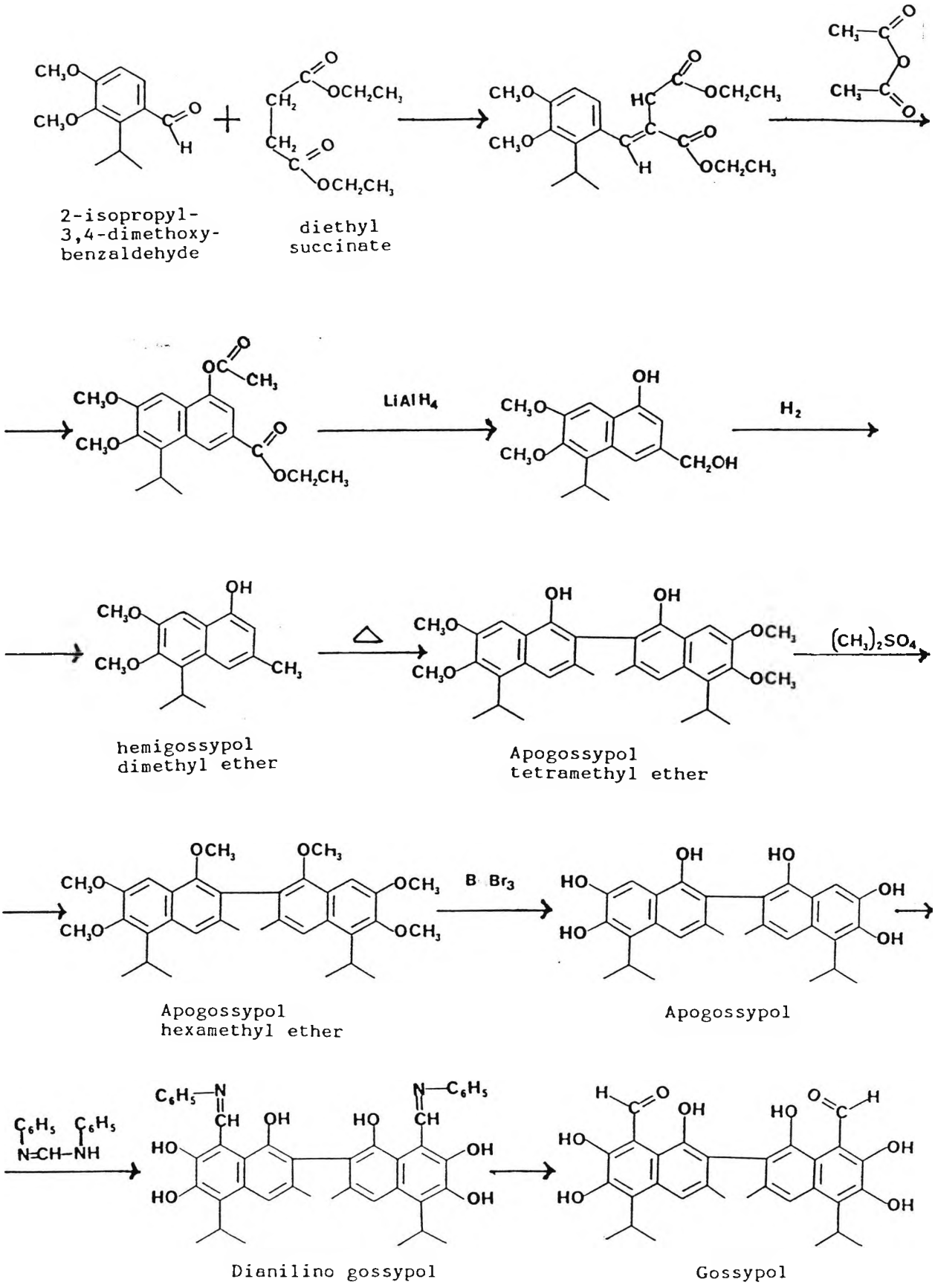
(9)



(10)

The α,α binaphthyl linked nuclei with methyl and isopropyl in the position 3,5 (compound 9) was favoured as many naturally occurring products with a naphthalene skeleton have methyl and isopropyl groups in positions 3,5 respectively.

The structure of gossypol deduced by Adams and coworkers was confirmed in 1956 by Edwards et al[15,25,26] through total synthesis (Scheme 2-5). Edwards et al used 2-isopropyl-3,4-dimethoxy- benzaldehyde as his starting material. After a condensation reaction and subsequent cyclodehydration, followed by reduction, he obtained hemiapogossypol dimethyl ether. Heating this product above its melting point forms a binaphthyl structure coupled at the position 2,2' corresponding to apogossypol tetramethyl ether. This confirms Adams and coworkers[13-21] elucidated structure of gossypol. On dimethylation and further treatment with N,N-diphenyl--formamidine, dianilino gossypol (4) is formed, which on hydrolysis yields gossypol (1).



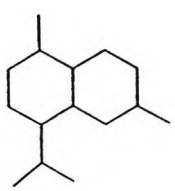
Synthesis of gossypol

Scheme 2-5

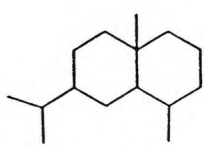
2.4 Biosynthesis of gossypol

The symmetrical binaphthyl structure of gossypol established by Adams and coworkers[13-21] is not compatible with any suggestion that this C-30 compound could be a triterpenoid derived in the usual way from squalene. Gossypol's structure strongly suggests that it is derived from two equal C15 sesquiterpene molecules with a decalin structure symmetrically coupled through phenols.

Two major classes of sesquiterpenoids have skeletal structures derived from decalin: cadinanes (11) and eudesmanes (12). Aromatic representatives of both classes have been found. Aromatic sesquiterpenoids of cadinane classes are quite numerous, since its skeletal structure is compatible with straightforward aromatization of either one or both of the rings. In contrast, aromatization of any precursor with the eudesmane skeleton is possible only with elimination or migration of the angular methyl group. This is obviously a less favourable process, and only two aromatized compounds of the eudesmane series have been found so far. Both sesquiterpenes are formed by alternative cyclizations of a chain of three isoprenoid units in normal head-to-tail arrangement.



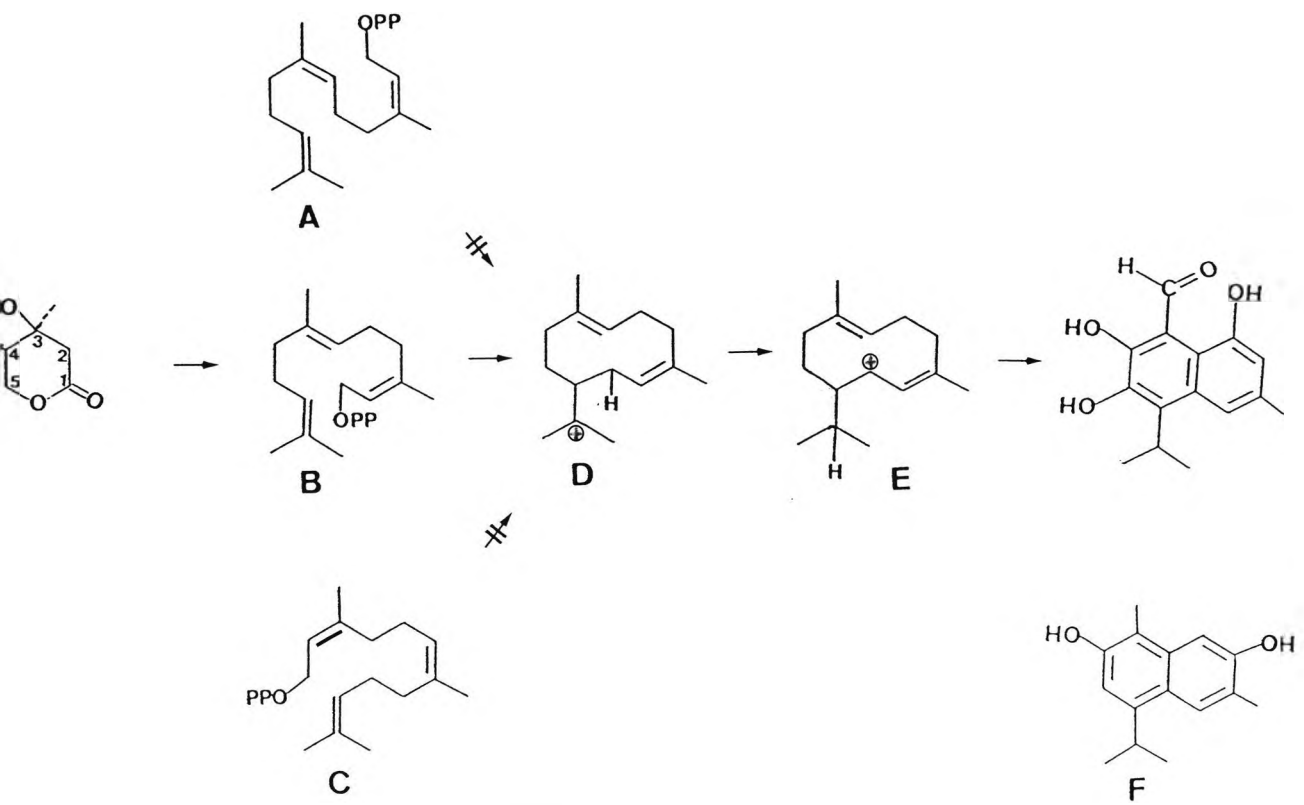
Cadinane class (11)



Eudesmane class (12)

In 1985 Arigoni et al[27] published their proof that the biosynthetic formation of gossypol in the cotton plant occurs, as with other investigated sesquiterpenes of the cadalene type, via ten-membered ring cations correlated by a

1,3-hydride shift (D -> E, Scheme 2-6). The biosynthesis of terpenes can be considered as an acetate based process. The first specific intermediate, the C-6 compound, mevalonic acid, is formed by three molecules of acetyl coenzyme A. An important intermediate in the biosynthesis of gossypol is the sesquiterpene, farnesyl pyrophosphate. It was recognized that the isoprene units in the farnesyl pyrophosphate could be folded in three different ways (A, B and C, Scheme 2-6) leading to different labelled decalin structures after cyclization. Arigoni et al proved in a radioactive labelled experiment, that gossypol was synthesized by the cotton plant by a cyclization mechanism through the 2-cis,6-trans folding pattern (B), followed by a 1,3-hydride shift (D -> E).[27]



Scheme 2-6

Pathway B ->D - >E (Scheme 2-6) has also been confirmed by Essenberg et al,[25] in a study of the biosynthesis of a related sesquiterpene, the 2,7-dihydroxycadalene (F), a cotton phytoalexin.

2.5. Biological activity

Cottonseed is a by-product of the cotton fibre and the processing of cottonseed has been a major industry in the cotton producing areas of the world for more than a century. The main valuable by-product is oil, used in the manufacture of edible products such as salad oil, margarine and shortening.

Harmful effects of cottonseed on animals were discovered in 1915 by Withers and Carruth.[29] Schwartze et al[30] correlated the toxicity with the free gossypol content of cottonseed.

Oil and meals obtained from cottonseed were recognized as highly desirable and since 1940 research efforts were concentrated in detoxifying the by-products of cottonseed.

Toxic effects of gossypol were minimized by binding free gossypol to proteins, where the reaction proceeds with participation of the aldehyde groups of gossypol and the amino groups of the amino acid.[31,32] Derivatives of gossypol and amino acids formed at 100°C are hydrolysed easily to the original starting material by alkalis, but under more extreme conditions of moisture and heat (115-130°C) a less easily irreversible condensation of gossypol with the amino acid occurs.

Under the influence of heat in the presence of oxygen and free ammonia, gossypol changes into gossypurpurin.[33] Gossycaerulin is found during the cooking of pulp at high temperature and acid media.[33] These gossypol transformation products by the action of heat and oxygen can be limited by lowering the temperature and by treating with anthranilic acid.

The specific deep brown colour of crude cottonseed oil is imparted not by gossypol itself, but by its transformation products and complexes with other constituents of the cottonseed.[34]

Until 1960, cottonseed meal was used principally as a protein supplement for ruminant livestock. When gossypol pigments were recognized as toxic to monogastric animals the use of cottonseed meal for feed was limited to swine and poultry. An alternative way of protecting the animals from the harmful effect of free gossypol was by the formation of complexes of gossypol with metals such as Fe(II), Fe(III) or Ca.[35] A sensitive indicator of the presence of free gossypol in feed is the development of a pink discoloration of egg yolks, when administered to laying hens. Addition of metal salts to the feed proved that complex formation was completely effective in preventing egg damage caused by gossypol.

Incaparina[36] and Peruvita[37] are vegetable protein dietary supplements containing cottonseed which were used in South America to alleviate human malnutrition. The composition specification guidelines for these protein supplements set up by the WHO/FAO/UNICEF Protein Advisory Group in 1964 were a maximum of 0.06% free gossypol and 1.2% total gossypol.[36]

2.6. Antifertility activity and toxicity of gossypol

The earliest account of infertility caused by consumption of cottonseed oil which contained gossypol was by the Chinese traditional herb doctor, Liu Bao-Shan in 1957. Liu Boa-Shan[38] observed that a village composed of more than 30 families had had no children for 10-15 years. All families had cooked during that period with cottonseed oil, which was the cheapest cooking oil available. Subsequently the mass production of soya bean oil made its price so much lower that the villagers quickly shifted from cottonseed oil to soya-bean oil. Quite unexpectedly, many of the wives of the 30 families began to conceive and have children. Liu Bao-Shan even speculated about the use of cottonseed oil for contraceptive purposes.

Unfortunately, Liu Bao-Shan's paper did not receive appropriate attention until the late 1960s, when many other parts of rural China experienced symptoms of fatigue, burning sensation and infertility. The peasants called this disease 'the burning fever' and by the late 1960s it had reached epidemic proportions. Doctors found that gossypol which remained dissolved in crude cottonseed oil was the cause of 'the burning disease' and advized their patients to stop pressing their own raw cottonseed oil.

Medical and scientific research workers from universities were sent to the affected areas to investigate. Epidemiological surveys confirmed that infertility was prevalent amongst the people suffering from 'the burning disease' and that gossypol was the cause.

While women seem to recover from infertility rather quickly, men only recovered depending on the intake of raw cottonseed oil. Therefore it was concluded that both time and

quantity played a part. Mortality was not observed as a result of "burning fever". Because the rate of recovery from male infertility was dependent on the individual amount of cottonseed oil a man had consumed, scientists conjectured that infertility would, most likely, be reversible if the gossypol dosage could be limited.

The discovery that gossypol ingested by men had reversible antifertility action without excessive toxicity led many Chinese scientists[39,40] in the 1970's to test the antifertility action of gossypol in animals. Gossypol was administered by different scientists in different degrees of purity in one of the three forms: gossypol, gossypol acetic acid or gossypol formic acid. The aim of the Chinese scientists like Wu, Wang, Lei and others was to study the mechanism of the antifertility action, reversibility and toxicity of gossypol. Their findings were presented at the "1st national conference on antifertility agents" in Wuhan in September 1972. An updated review was published in 1984.[40]

Pronounced differences were observed amongst animal species in their sensitivity to the antifertility action of gossypol.[39] Amongst the laboratory animals tested, hamsters seemed to be the most sensitive, followed by rats, monkeys and dogs in decreasing order, while rabbits and mice appeared to be insensitive.

The effective dose for the hamster was 5-10mg/kg/day, given for 6-12 weeks; recovery of fertility occurred 4-14 weeks after withdrawal of gossypol. The effective dose for rats ranged from 10-30 mg/kg/day, given for 3-10 weeks. Recovery of infertility was dose-dependent, occurring 3-12 weeks after withdrawal. Gossypol at a dose of 7.5 mg/kg/day administered for longer periods of time also induced

infertility in rats, but 6 mg/kg/day given for 6 weeks was found to be ineffective. It was found that long-term treatment caused complete atrophy of the seminiferous epithelium in some animals and consequent sterility. Dogs were sensitive to the antispermatogenic action only when gossypol was administered at toxic doses. Rabbits were completely insensitive to the antispermatogenic action of gossypol but very sensitive to its toxic effects, resulting in eventual death. The response of mice to gossypol was similar to that of rabbits. Monkeys were found to be moderately sensitive to the antispermatogenic action of gossypol. The conclusion of the animal studies was that gossypol itself is antispermatogenic and the effect was not due to other impurities present in the crude preparation of cottonseed oil.[40]

Gossypol given orally was absorbed through the intestine as well as through the epithelial lining of the stomach. Fecal excretion was the major route by which gossypol administered either orally or parenterally was removed from the animal body. Most of the absorbed gossypol was excreted via the bile, suggesting biliary circulation of gossypol between the liver and the intestine.[40]

Elimination of gossypol from the body is slow. It takes a rat 19 days to eliminate 97% of the dose from its body. Continued administration therefore would lead to accumulation. The order of gossypol distribution through the bodies of all animals is liver, gastrointestinal tract, spleen, lymph nodes, kidney, heart, lungs, pancreas, salivary glands, muscle, adipose tissue, testes, blood, urinary bladder, brain and spinal cord. Because gossypol concentration is high in the liver and kidney, there was a

special concern about toxic effects to these organs. However, the Chinese scientists believed that because of the regenerative and compensatory properties of the kidney and liver relatively little harm would be done to these organs.[40]

The first clinical trial of gossypol as a male antifertility agent was carried out by Qian et al[41] in 1972 on 25 volunteers. They found that gossypol given orally at a dose of 60-70 mg/day for 35-42 days caused a gradual increase in the percentage of non motile spermatozoa in the ejaculate, followed by oligospermia, necrospermia and azoospermia in all 25 volunteers. Sperm motility decreased markedly as early as the second week of administration, encouraging the theory that gossypol may act on epididymal as well as testicular spermatozoa. Recovery occurred around three months after withdrawal. The side effects of this dosage were reversible and generally of mild degree, mainly including decrease or increase in appetite, fatigue, dryness of mouth, diarrhea and tendency to sleepiness; very few complained about decreased libido and there was an insignificant depression of serum potassium levels. When the dose was decreased to 24-35 mg/day, the duration of the treatment had to be correspondingly prolonged, and the side effects were significantly reduced. Qian et al[41] concluded that gossypol was an effective antispermatogenic agent in men and in all his volunteers the health status was reasonably good after 1 year. They advised however, that the significance of the side effects needed further toxicological studies.

In 1973 a multicentre clinical trial of gossypol was organized. 3 different types of tablets containing gossypol, gossypol acetic acid and gossypol formic acid were prepared using "highly purified compound". The clinical trial with

gossypol started in 1974 in 14 provincial and municipal districts of China, and by 1980 the total number of volunteers had amounted to 8806.[42] The optimal loading and maintenance doses were determined through initial years of trial and readjustment. The loading dose was finally set at 20 mg/day given for 60-75 days, followed by a maintenance dose of 50 mg/week. The overall antifertility efficiency was claimed to be 99.07% as estimated by sperm examination. Common side effects observed included fatigue (12.6%), gastrointestinal symptoms (7.4%), decreased libido or potency (5.0%), dizziness (3.88%), and dryness of the mouth (3.1%). Fatigue was thought to be related to potassium deficiency and the Chinese scientists reported that administration of potassium alleviated fatigue. Additionally, an infrequent but serious side effect, hypokalemic paralysis, was uncovered during the expanded trials, the overall occurrence being 0.75% (66 volunteers). In certain districts it was as high as 4.7%. Through later studies of hypokalemia (decreased serum potassium level) it was postulated that the occurrence of hypokalemia was due to a regional effect correlating with the low dietary intake of potassium. The dietary intake of potassium was later analysed and calculated to be 26.9 mEq/l in a hypokalemia high occurrence region compared with 53.2 mEq/l calculated for a region with no cases of hypokalemia.[43] It was said that most of the hypokalemic cases recovered soon after potassium repletion, but a few only recovered after prolonged potassium therapy.

The results of the antifertility action of this clinical trial were similar to those reported by Qian et al,[41] but taking longer to reach infertility as the dosage was much lower. It took 2-3 months to reach oligospermia (sperm count

below 4 million/ml). Recovery occurred 3-4 months after withdrawal of gossypol in most cases.

Semen analysis was followed up in 2067 volunteers for 6 months to 4.5 years after cessation of gossypol administration. This involved sperm counts and determination of sperm motility. It was concluded that 90.1% had reversible infertility from which 73.7% had a sperm count over 4 million/ml, the remaining 16.4% had appearance of sperm, though the sperm count was below 4 million/ml. The remaining 9.9% of the volunteers remained azoospermic. It was observed that volunteers being dosed for less than 2 years had a recovery much higher than those taking it for more than 2 years.[42]

Electron microscopic examination of spermatozoa in gossypol-dosed volunteers revealed damaged and ill developed acrosomes, which in some cases disappeared completely. The sperm head nucleus showed a less condensed nucleoplasm. A derangement of the spiral mitochondria cristae was also observed. Histochemically, a decrease in succinic dehydrogenase and lactic dehydrogenase was measured in the midpiece.[42]

After discontinuation of gossypol treatment, the spouses of 266 volunteers became pregnant. The 53 spouses who continued pregnancy gave birth to apparently normal babies.[42]

It was a difficult task to correlate the data of the 15 hospitals, as no uniformity of protocol relating the subjective symptoms was followed in these clinical trials. Another factor was that not all subjects were dosed with the same form or degree of purity of gossypol. No entry criteria such as age group, fertility, or histochemical values was set up in the clinical trials to admit volunteers, nor were the

dosed cases followed parallel to a control group. Therefore the clinical trial results did not carry statistical significance and few valid conclusions could be drawn.

Based on the findings of the animal studies and the multicentre clinical trial of gossypol, early in 1980, a scientific group belonging to the China National Coordinating Group of Male Antifertility Agents recommended to the Chinese Ministry of Health that gossypol pills should be introduced for general use. There was a strong opposition by another group of Chinese scientists concerned with the unresolved safety and toxicity issues and they recommended that further research should be carried out.

Simultaneously in 1980, the Western countries showed a great interest in gossypol as a potential male contraceptive, as its action was reported to be independent of the hormonal events of the hypothalamo-hypophysial-gonadal axis. The reported side effects were very mild compared with those associated with the experimental use of hormonal steroids as a male contraceptive. The use of androgens, progestins, or combinations of the two by men were associated with breast tenderness, gynecomastia, weight gain, loss of libido and changes in blood chemistry as a consequence of altered liver function.

At the end of 1981 a new clinical trial funded by the Rockefeller Foundation was initiated by Liu[43] in the Capital Hospital Beijing, recruiting 152 men. All of them met entry criteria. To follow a double blind procedure, the volunteers were split in two groups: 75 men received 20 mg gossypol for 75 days, the other 77 men took a placebo for the same period. Both groups of men were similar in the initial biochemical parameters prior to dosing.

The double blind trial was useful in studying fatigue, decreased libido and appetite changes. There was a follow up on day 37 and day 75, where body weight, blood pressure, haemoglobin, serum potassium, sperm count and electrocardiograms were measured. After 75 days of gossypol treatment, 31% were azoospermic, 61% oligospermic (less than 4 million sperm /cc), 3% had a sperm count between 5-9 million/cc and 5% higher than 10 million/cc. Out of this 5% (3) higher than 10 million, 2 volunteers "forgot" to take gossypol pills for 2 weeks. This trial established an efficacy rate of 92% for a sperm count <4 million/cc. No statistically significant difference could be found between both groups in the incidence of fatigue, loss of libido or gastrointestinal disturbances. Also no differences were observed in serum K, body weight or blood pressure between the gossypol and placebo treated groups.[43]

Liu[43] speculated about the earlier multicentre Chinese study that fatigue may be an early symptom of impending hypokalemic paralysis. He suggested that the relationship between hypokalemia and fatigue could be correlated by studying the difference in the serum K level between those gossypol treated males who had complained of fatigue and those who did not suffer from fatigue.

Two additional clinical trials were carried out in Austria[44] and in Brazil,[45] without control groups. In 1981, 5 subjects underwent clinical trial in Austria. Three were aged 60-65, the remaining two were 47 and 48 years old. The gossypol loading dose was 20 mg daily for 3 months. The aim of the study was to follow the effect of gossypol on the tubular compartment in human males, and the hormonal and electrolyte status. In the 2 younger men, sperm counts were

measured repeatedly and azoospermia was reached at the 8th week of gossypol treatment, and continued for at least 2 months after cessation of the treatment. Testicular biopsies in the 3 older men revealed an almost complete arrest of spermatogenesis in the 10-12th week of the treatment phase.[44] The parameters represented in figures 3 and 4 were studied. The semen volume did not change with gossypol treatment, while sperm density was reduced to zero within 3 months and the subjects remained azoospermic for 2-4 months after cessation of treatment. Citric acid and fructose levels (both indicative of androgenic status in the semen) were not affected by gossypol treatment, and this was further corroborated by the testosterone level also being unaffected (Figure 2-3).[44,45] Gossypol action was demonstrated to be independent of the pituitary gland. The gonadotrophins FSH and LH and also estradiol (E2) remained within normal ranges during treatment (Figure 2-4).

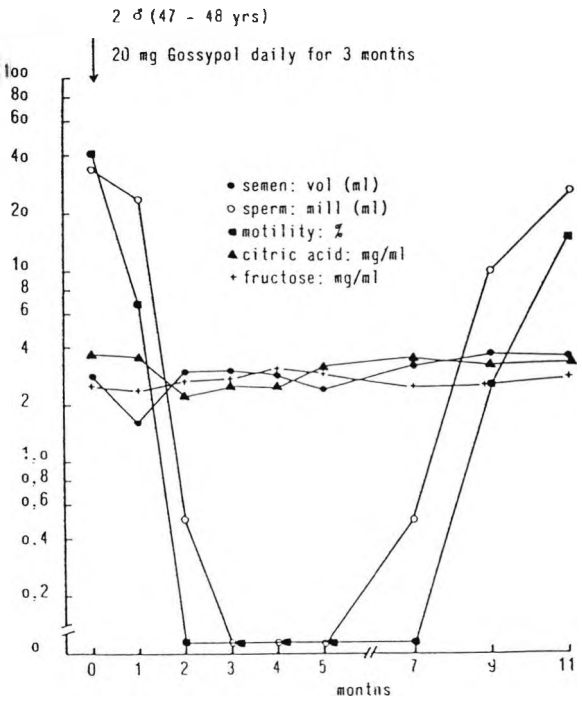


Figure 2-3
Sperm analysis data in 2 men.[44]

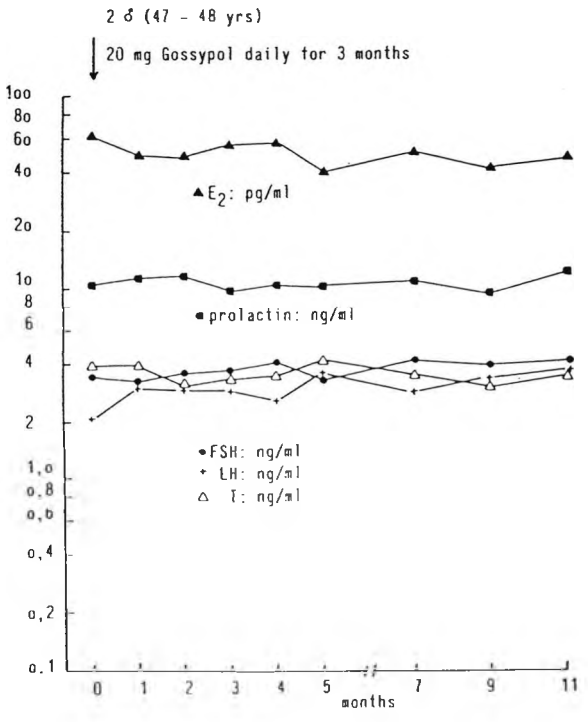


Figure 2-4
Hormone values for testosterone LH, FSH, E_2 and prolactin.[44]

Another clinical trial was carried out in 1984 in Brazil.[45] Twelve subjects were selected and treated for 4 months with a daily loading dose of 20 mg of gossypol acetic acid, and 60 mg weekly maintenance dose for a further 4-6 months. A 20% reduction in sperm count was found in the first month and by the end of the 4th month 10 subjects were azoospermic and 2 subjects had severe oligospermia (0.2-0.5 million/ml) with necrospermia. Following discontinuation of gossypol the sperm count returned to the original pre-treatment level in 50% of the subjects, in 20% it rose to 50% of the original pre-treatment level, remaining there constant for as long as 2 years. 30% stayed azoospermic for at least 2 years. This Brazilian clinical trial proved again that the antifertility action of gossypol is independent of action on the hypothalamo-hypophysial-gonadal axis and no change in libido or frequency of intercourse was reported. Only one subject complained of fatigue. The action of gossypol in man was located in the testis affecting spermatogenesis in the seminiferous tubules and epididymal sperm motility.[45]

A large programme of in vivo animal experiments and in vitro research has been carried out worldwide to study the mechanism of action of gossypol and its possible toxicity in different organs.

Kalla et al[46,47] administered 2.5 mg/kg/week of gossypol acetic acid for 3 months to 4 adult bonnet monkeys, using 2 further monkeys for control. They observed a marked decline of sperm count on the 75th day of administration, similar to the observation of Shandilya and his associates,[48] of gossypol in male cynomolgus monkeys. Sunderam[49] however did not observe significant changes in sperm count/ejaculate in

the rhesus monkey treated with gossypol at doses of 5, 20 and 80mg for 10-12 weeks. This discrepancy in Sunderam's observations seems to be due to his use of peanut butter as the vehicle for administration of gossypol. It was suggested that gossypol binds to lysine and this bound gossypol is not biologically active thus explaining the lack of antifertility action. Alternatively, the rhesus monkey used by Sunderam might be resistant to gossypol's antifertility action.

The relation between effective and toxic doses of gossypol was researched by various scientists.[50,51] The usual manifestations of toxicity are depressed appetite, loss of body weight and inefficient protein utilization.

It was already reported that different animals showed different sensitivity to gossypol's antifertility and toxic effects. Chang and Segal[50] evaluated antifertility and toxicity activities of gossypol acetic acid in different strains of the same animal (rats). Reduced growth rate of the rats was taken as an indication of toxicity. It was observed that 4 different strains of rat had a different growth rate versus their control group, establishing that gossypol can affect differently different strains of the same animal.

Chang et al[51] studied the extent of antifertility effect in rats administered 7.5 mg and 15 mg/Kg of gossypol acetic acid for 4, 8 and 12 weeks and 30 mg/Kg of gossypol acetic acid for 6 weeks. The spermatozoa collected from the cauda epididymis at the end of the gossypol treatment showed abnormal motility and morphology. Morphological abnormalities included sharply bent tails and complete separation of the tail from the head. All dose levels reduced the total number of sperm to less than 50% compared to those in control

animals, and most of the sperm from treated animals were completely immotile apart from some sperm of the low dosed rats. The number of spermatozoa with normal appearance decreased progressively with the increasing dose while the numbers with bent tails and separated heads increased. There were no significant differences in the weights of testes, epididymis, seminal vesicles, and prostate,[47,51] nor in liver, spleen, kidney, thymus, adrenals, thyroid or pituitary of the rats treated with 7.5 or 15 mg/Kg gossypol for 12 weeks compared with control animals. However, gossypol at high doses, 30 mg/Kg, reduced significantly the weight of epididymis, seminal vesicles and prostate, though no change was found in testicular weight.

Huang et al[52] studied the action of gossypol on the rat heart. They found that action potentials were reduced in ventricular cells, and in 80% of the hearts exposed to gossypol for 30 minutes or more (using an organ perfusion technique after removal of the heart into an organ bath), ventricular tachyarrhythmia were observed.

Different autoradiographic studies with ^{14}C-gossypol showed a concentration of radioactivity in the testis in the interstitial cells and in certain cells of the seminiferous tubules, while in pituitary cells and hypothalamic neurons no concentration of radioactivity was seen.[53,54] These results suggested that there is no central site of action for gossypol and that the primary targets for gossypol action are testicular interstitial cells and certain cells of the seminiferous tubules. In 1988 Stumpf et al[55] also observed accumulation of labelled gossypol in testis, kidney and liver, while there was little in brain, pituitary and

epididymis. In testis, high accumulation occurred in interstitial cells, with low levels in Sertoli cells, spermatogonia and spermatocytes.

Both testicular and epididymal germ cells exhibit morphological defects after gossypol is administered to rats daily for 3 weeks.[53,56] These abnormalities include structural defects of the sperm head, degeneration of the cell plasma membranes and mitochondria, and disarrangement and/or absence of one or more of the tail's outer dense fibres.

In the step 18 to 19 spermatids, detrimental effects of gossypol are evident in the form of nuclear vacuolation, mitochondrial swelling and dismembranation. In maturing spermatids detachment of the acrosome is seen.[56,57] Whole body autoradiographic studies after administration of [^{14}C] gossypol showed that the drug is incorporated into the liver, lung, kidney, heart, fat and testis.[54] However the testis is the only organ to show discernible damage at lower doses of gossypol.

No defect was found in the epididymal epithelium although abnormal seminiferous tubules were always seen, the severity of the damage never exceeding 46%, even at the highest dose.[53] Ultrastructural examination demonstrated that the cellular basis of this damage to the seminiferous tubules is the presence of inter- and intra-epithelial vacuoles occurring primarily, though not exclusively, in the Sertoli cells. The biochemical damage to Sertoli cells cannot be the only explanation of the antifertility action of gossypol, as in vivo the mitochondrial sheath abnormalities and the decrease in sperm motility occur well in advance of the Sertoli cell changes.[53]

From the data of the in vitro study on bull spermatozoa,[57] sea urchin sperm,[58] mouse testes,[59] boar ejaculated spermatozoa,[60] and arbacia sperm,[54] it has been conjectured that gossypol may act on spermatogenesis by interruption of the energy chain in the spermatozoa. The motility arrest may result from a reduction in energy supply through inhibition of lactate dehydrogenase X-(LDH-X), pyruvate dehydrogenase and the succinate to cytochrome C segment of the electron transport chain. Oxidative phosphorylation and mitochondrial Mg^{2+}-ATPase are also suppressed by gossypol.

The experimental results of Ueno et al,[61] published in 1988, also support the thesis that gossypol inhibits sperm motility by blocking ATP production and utilization; gossypol acts on the mitochondria, suppressing oxygen consumption, inhibiting pyruvate dehydrogenase and ATPase activity and on the motility apparatus by preventing protein phosphorylation. The nature of the chemical interaction of gossypol and macromolecules was speculated to be through Schiff's base formation and by hydrophobic attraction.

It has been demonstrated that gossypol inhibits enzymes of the glycolytic pathway and the respiratory chain and uncouples oxidative phosphorylation. The net result is a reduction in ATPase synthesis.[58]

A study by Lee et al[59] found that amongst the isozymes of lactate dehydrogenase, lactate dehydrogenase-X from mouse and human was selectively inactivated by gossypol, the degree of inactivation depending on time, concentration, pH and buffer.

Tso[60] studied the relationship between sperm motility, energy depletion measured by ATP content, and gossypol concentration. Sperm motility fell much more rapidly than did sperm ATP content after dosing, indicating that the effect on ATP is not the sole (or primary) factor influencing motility.

The formation of a chelated complex 2:1 between gossypol and Mn^{2+}, as found in electron spin resonance studies, was linked with the inhibition of sperm adenylate cyclase, which is essential for maintaining high levels of cAMP in sperm and, in turn, motility.[62]

It is also believed that gossypol affects cyclic AMP production at the level of ATP conversion to cyclic AMP.[59,63]

^{14}C-gossypol binds to the membrane of arbacia sperm via a covalent linkage. The bonding is postulated to be between the aldehyde group of gossypol and the primary amines of proteins by formation of a Schiff's base. Moreover, bonding may occur by other mechanisms such as oxidation of phenolic groups forming reactive quinones, hydrogen bonding, Van der Waals interactions and charge transfer complex formation. Gossypol must be able to penetrate through the membrane to act on the mitochondrial sheath.[54]

In 1988 Fei and Teng[64,65] described the effect of gossypol on nuclear proteins in rat testis, observing a reduction of basic proteins in the spermiogenic cells. The inhibitory effect of gossypol is more prominent in the elongating spermatids than in the round spermatids. The possible risk of damage to other germ cells (spermatogonia and spermatocytes) by gossypol interfering with the proteins in gene regulation is still under study.

Corin et al[66] created a model to 'explain' the different behaviour of gossypol to a target (testicular cell) and non target. Gossypol enters the cell by a partition mechanism, i.e. unmediated diffusion. Gossypol, being hydrophobic, may be very soluble in membranes. It also binds to extracellular protein with high affinity. Corin et al speculate that protein bound gossypol is not a substrate for unmediated diffusion into the plasma membrane, but rather that free gossypol is such a substrate. Once free gossypol is dissolved in the lipid bilayer of the plasma membrane, gossypol may interact with membrane proteins, internal membranes or other intracellular targets. The capacity of a cell to accumulate and retain gossypol may therefore be determined by the membrane composition of a cell. In a particular target cell, e.g., in the testis, there may be a specific binding component with an affinity for gossypol greater than that of serum proteins or non target hydrophobic compounds of the plasma membrane. This would facilitate concentration and retention of gossypol in the target tissue with subsequent pharmacological action.

Teratology experiments on rats treated with gossypol proved that in the 1st and 2nd generation there is no toxic action on the embryonic development and that the incidence of malformation in fetuses (0.2-0.3%) is lower than the spontaneous incidence expected of 0.5%.[67,68]

Whereas racemic gossypol is produced by the cotton plant (gossypium species of the family Malvaceae), (+)-gossypol has been isolated from another member of this family (Thespesia populnea).[69-71] (+)-Gossypol does not form an acetic acid

complex, unlike gossypol racemate.[69] (+)-Gossypol had proved to be inactive in antifertility tests in animals. These findings were confirmed by antifertility experiments on rats and on hamsters.[72-74]

In 1985 pure (-)-gossypol obtained by chromatographic separation by Matlin et al[75] proved to be an active male antifertility agent in hamsters.

Wang et al[76] studied the fertility of male rats treated with (+)-gossypol and (-)-gossypol. They concluded that (+)-gossypol at the dose administered had no toxic effect on the body weight and also did not affect the motility of the sperm in the cauda epididymis nor alter the fertility of the rat. At an equal dose, racemic gossypol caused loss of fertility. (-)-Gossypol, administered at half the dose of the racemate, did cause loss of fertility of the male rats and the sperm in the cauda epididymis were found to be dead. Toxic effects were observed, reflected in loss of body weight, in a few cases ending in death.

Several in vitro studies were reported on the racemate and on both enantiomers of gossypol to elucidate the mechanism of antifertility action. Liu et al[77] studied the effect of the enantiomers on the mitochondrial LDH-X of human and guinea pig sperm. They observed that when sperm was incubated for 24hr at a concentration of 10 µg/ml, enzyme activity in (+)-, (±)-, and (-)-gossypol groups declined by approximately 5%, 33% and 44%, respectively. When the concentration of gossypol was increased to 50 µg/ml, (-)-gossypol could inhibit the enzyme activity almost completely (80-90%). Liu et al suggested that (-)-gossypol exerts more inhibitory effect on sperm LDH-X activity than (+)- and (±)-gossypol.

Yao et al[78] described an in vivo experiment on the effect of (+)-, (-)- and (±)-gossypol on rat testicular LDH-X, which showed no significant differences in inhibition, neither was there found to be a difference between the treated and the control animals. Yao concluded that the antifertility effect of gossypol could not be explained on the ground of inhibition of LDH-X as observed in vitro.

The study from Sufi et al[79] suggests that both optical isomers of gossypol exert similar dose related effects on both basal and LH-stimulated testosterone released from dispersed mouse Leydig cells. It would thus appear that the effect of gossypol on spermatogenesis may be highly stereospecific whilst its effect on Leydig cells and sperm in vitro might be caused by less stereoselective mechanisms.

From a series of gossypol derivatives studied, Sonnenberg et al[80] in 1988 concluded, that the carbonyl groups of gossypol are needed for the inhibition of erythrocyte anion transport and although the hydroxyl groups have some effect, they are not essential. Of the more simple structures related to gossypol, those that were active in cytocidal and spermicidal assays, were bi-aromatic, linked by a 1- and not a 4-carbon chain which had free phenolic hydroxyl groups.

In the search for an analogue of gossypol which would retain the antifertility activity while eliminating its pharmacologically undesirable properties, Hoffer et al[81] in 1988 tested 14 new synthetic analogues, finding none of them to be active as a male contraceptive. One of these inactive

analogues was "diethylgossypol", in which the two i-Pr groups were replaced by Et. This suggested that intact isopropyl moieties may be essential for gossypol's antifertility properties.

During the course of studies on the mechanism of action of gossypol, it was observed to be a potent inhibitor of the lactate dehydrogenase enzyme LDH which reduces pyruvate to lactate under anaerobic conditions. This observation led to investigations to establish whether gossypol had antiproliferative effects upon tumour cells. One feature known to distinguish malignant from normal mitochondria is that the oxidoreductase enzyme formed in tumours will favour anaerobic conditions. Lactate dehydrogenase is an oxidoreductase present in the glycolytic pathway. It was demonstrated that gossypol possesses in vitro antiproliferative activity at concentrations used for contraceptive purpose in humans [82-85]. Haspel et al[83] suggested that the relative lack of side effects during in vivo administration of gossypol as a contraceptive probably reflects a buffering action by serum protein. Serum and albumin have been demonstrated to inhibit the cytotoxic activity of gossypol.

Joseph et al[86] examined the cytotoxicity of the enantiomers and the racemic mixture of gossypol on normal and tumour derived cells in culture. They observed that the (-) enantiomer of gossypol was more cytotoxic than the (+) form. To bring about a 50% reduction in the cell growth it was necessary to use a mean concentration of 1.9 $\mu g ml^{-1}$ for (-)-gossypol compared to 12.7$\mu g ml^{-1}$ for the (+)-form and 6.2 $\mu g ml^{-1}$ for the racemic mixture. At a lower concentration gossypol inhibits cellular proliferation, at higher

concentration gossypol brings about complete cell lysis. It was observed that the biochemical effect of gossypol occurred rapidly after addition even though cellular changes may not be obvious for some hours. Gossypol, once added to the growth culture fluid, lost cytotoxic potential through binding to plasma proteins. The experiments revealed that to obtain a 50% inhibition in growth, about 100 times more gossypol was necessary in the presence of 100% serum than in 1% serum. Gossypol was observed not to have a cytotoxic effect on bone marrow or liver. Various scientists postulated that this effect lay in the protection afforded by higher protein concentration. Most cells are perfused not by plasma but rather by a fluid that is an ultrafiltrate of plasma formed at the blood capillaries, which has a protein concentration that is less than one quarter that of plasma. There are exceptions such as liver and bone marrow, whose interstitial fluid is effectively plasma.

Band et al[87] also studied the antiproliferative effect of gossypol and its optical isomers on various human cell lines, and concluded that (-)-gossypol was 3.6-12.4 times more potent than (+)-gossypol and 1.48-2.65 times more potent than (+/-)-gossypol. The most sensitive indicator of gossypol action was a decrease in DNA synthesis, followed by inhibition of protein synthesis and uptake of rhodamine-123 by mitochondria as tested in an ovarian cancer cell line and a fibroblast line.

Benz et al[88] studied antiproliferative activities of gossypol on six human cell lines derived from breast, pancreas, prostate, colon and cervix carcinomas and compared them with another known antimitochondrial agent, rhodamine-123. While both have a good antiproliferative

activity on the carcinomas studied, 10 µmol of gossypol shows little growth inhibition of human marrow compared to total inhibition of stem cell growth of human marrow by 10 µmol of rhodamine-123. Benz et al also observed in human breast cancer cells that 11 days after treatment with 10 µmol of (-)-gossypol no cell growth was observed compared to 91% cell growth with 10 µmol of (+)-gossypol. In 1988 Benz et al[89] published a technique to measure the decline in tumour ATP levels occurring within 24 hours of treatment with gossypol by [^{31}P]-magnetic resonance spectroscopy, suggesting that this non invasive technique may serve as an early biochemical monitor of gossypol toxicity.

Gossypol was also found to be a powerful inhibitor of the NAD-linked enzymes α-hydroxyacid dehydrogenase and maleate dehydrogenase of the flagellate parasite Trypanosoma cruzi which causes Chagas' disease. The catalytic properties of these NAD-linked enzymes resemble those of the mouse and rat LDH X. Since there is presently no satisfactory drug for use in Chagas' disease, a very serious endemic illness affecting millions of people in South America, gossypol can be considered as a promising pharmaceutical agent.[90]

In October 1986 a Symposium entitled "International Symposium on Gossypol Research for Fertility Regulation" was organized in Wuham, China, to review all the aspects of the research studies related to gossypol. The papers presented at this symposium were published in the February and March 1988 editions of CONTRACEPTION. Clinical experience identified two specific items of concern; these were hypokalemia and irreversibility of infertility.

The incidence of hypokalemia in men taking gossypol was found to be variable (from less than 1% to 10% according to

the region).[91] The evidence suggests that the hypokalemia may be related to impaired renal function[85] and renal tubular damage[92] which in most cases is reversible. Neither supplement of potassium salts nor a potassium excretion blocker prevented the effect of gossypol on serum potassium levels, contrary to previous claims.[93] The examination of the inhibitory effect of gossypol on the activity of (Na^+ + K^+)-ATPase showed that while gossypol is noncompetitive with ATP, Mg^+, Na^+ and K^+, it is a specific and potent membrane active agent;[94,95] the penetration and injury of the cell membrane by gossypol inhibited Na^+- and K^+-ATPase activity and consequently the K^+ efflux was increased, offering an explanation to the possible mechanism of the development of hypokalaemia.[94]

The incidence of irreversible testicular damage, defined as either azoospermia or severe oligospermia, was found to vary between 15% and 50%, depending on the dosage administered, period of treatment and time elapsed since treatment was ended.[96,97]

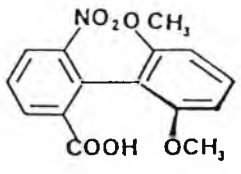
2.7 Optical activity of gossypol : atropisomerism

Gossypol is a chiral molecule by virtue of restricted rotation about the internaphthyl bond. Optical isomerism due to restricted rotation about a single bond, where the isomers can be isolated, is called atropisomerism.

In 1921 Kenner synthesized 6,6′-dinitrodiphenic acid. The study of the optical activity of this compound led Kenner in collaboration with Christie in 1922 to establish the first correct rules about atropisomerism. Initially, Kenner wrongly identified the two acids as geometric isomers, based on an erroneous theory from Kaufler, which situated both rings of the biphenyl compound in parallel planes. Kenner also wrongly believed that his acid was the trans isomer since on reduction it gave a dilactam. In 1922, Christie and Kenner resolved the acid, obtaining a value of $[\alpha]_D^{25} = +225°$. They explained the optical activity by a novel theory: a coaxial structure would also be disymmetric provided that both benzene rings were not co-planar.

In 1926, Turner and Le Fe′vre, Bell and Kenyon, and Mills simultaneously postulated that the biphenyl molecules had the two benzene rings colinear, and that the introduction of ortho substituents would prevent free rotation of the nuclei about the coaxis. The impingement of the o-substituents would prevent the two benzene rings being coplanar, producing coaxial twist. The non planar biphenyl molecules are disymmetric.

It was soon realized that the introduction of bulky o-substituents in a biphenyl molecule was insufficient to have an optical compound. When the compound had a vertical plane of symmetry, resolution was impossible as with the 2-nitro-6-carboxy-2′-6′-dimethoxybiphenyl (13).



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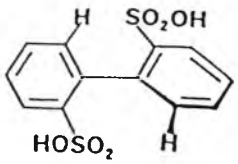
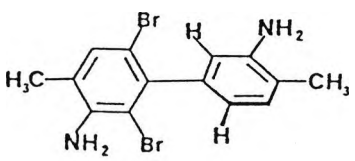


(14)

Initially it was believed that at least three ortho substituents were necessary to confer optical activity on a biphenyl molecule. In 1932, Leslie and Turner resolved a diortho substituted biphenyl molecule, (14), where the two large o-substituted sulphonic acid groups were impeded by only two small hydrogen atoms.



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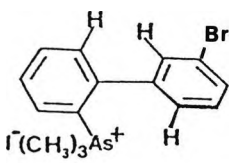
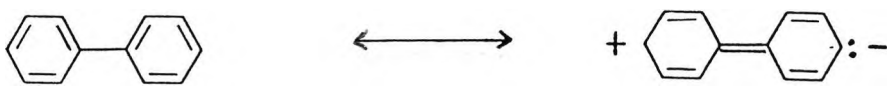


(16)

In 1935, Patterson and Adams resolved ortho disubstituted biphenyl molecules, where both bulky ortho substituents were in just one ring, such as 2,6-dibromo--3,3′-diamino-4,4′-dimethylbiphenyl (15). These compounds racemized easily on heating. In 1933, Leslie and Turner observed the mutarotation of a very bulky orthomonosubstituted biphenyl, which due to its high rate of racemization could not be resolved. This compound is the (+)-camphorsulphonate of 3′-bromobiphenyl-2-trimethylarsonium iodide (16).

Ultraviolet studies of the atropisomers were particularly instructive to demonstrate that both nuclei were non coplanar in the biphenyl derivatives. Biphenyl itself shows a strong UV absorption max at 249 nm (Emax = 17,000)

due to extended resonance through both linked benzene rings, involving an inter-annular conjugation (Scheme 2-7). It has been demonstrated that the resonance energy of the biphenyl depends on the angle of twist of the linking bond in a square cosine relationship.



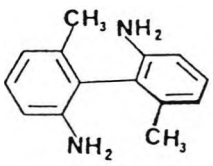
Scheme 2-7

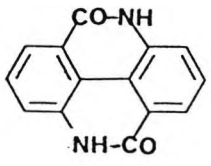
Biphenyl is only planar in the crystalline state as the lattice forces increase with the planarity of the compound. X-ray studies of crystalline biphenyl show that it is a planar molecule. In any other physical state, steric repulsion of the ortho hydrogens atoms caused a coaxial twist, the two benzene rings in the biphenyl being inclined to each other at approximately 45°. Resonance is not severely inhibited until the interplanar angle becomes quite large. The introduction of ortho substituents in the biphenyl molecule twist the phenyl rings further apart, between 60-90°.

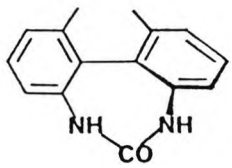
Similarly, just the introduction of the ortho-methyl substituent shifts the conjugation band to a shorter wavelength (hypsochromic shift), 236.5 nm, simultaneously reducing the intensity of the conjugation band. Introduction of additional ortho substituents leads to a progressive disappearance of the conjugation band. The presence of one or more bulky substituents would be enough to completely inhibit the conjugation band.

To further develop the obstacle theory based on the non coplanarity of optically active biphenyl derivative (17), Meisenheimer published in 1927 his observations after linking

together the ortho substituent interfering groups. He observed that if the ortho substituents were linked by condensation reactions forming part of a six member ring (18), both nuclei of the biphenyl would be forced into a coplanar arrangement, inhibiting completely the optical activity. If the ortho substituent were linked by a bridge forming a 7 member ring (19), that extra flexibility would allow two nuclei to remain non coplanar and to keep their optical activity.

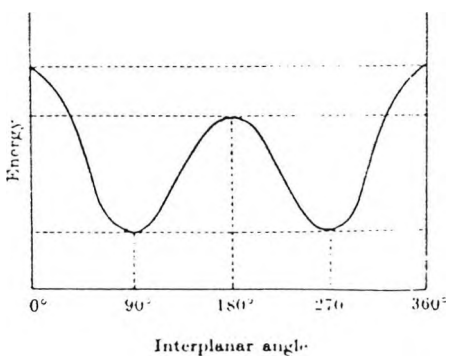






(17) (18) (19)

The interconversion or racemization of biphenyl atropisomers involves passing through a planar maximum energy conformation. The larger the ortho substituents in a biphenyl molecule, the more difficult it will be to obtain a planar transition state due to overcrowding, and therefore less likely racemization. Thus, the racemization energy diagram of a biphenyl is as in Fig. 2-5, the bulkier the groups the higher the energy barrier.



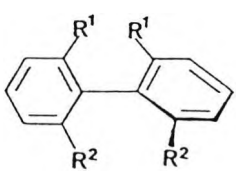


Fig 2-5: Energy diagram for racemization of biphenyls

It has been observed that biphenyl molecules containing equal size opposed ortho substituents have a higher energy barrier and lower racemization than those containing unequal size ortho substituents.[98] Adams and coworkers[99] studied extensively the racemization of tetra-substituted biphenyls. They observed that when at least 2 out of 4 o-substituents are bulky, very stable enantiomers can be resolved. Biphenyl molecules containing medium size o-substituents can be resolved, but they racemize easily by warming in a suitable solvent. Biphenyl molecules containing very small o-substituents, such as $-OCH_3$, -F, -H racemize so easily that resolution is not possible.

When biphenyl ortho substituted atropisomers pass the planar transition state, the interfering o-substituents can slip past each other by bending the bonds away from each other. This o-substituent bending is prevented when the neighbouring position (either 3 or 5) is occupied by a bulky group, creating a buttressing effect. The buttressing effect is reflected in the increase of the transition state energy, which increases with the increase of the size of the neighbouring substituents. Buttressing makes the enantiomers more stable and more difficult to racemize.

Optically active biphenyls do not normally contain asymmetric atoms. To attribute to the biphenyl atropisomers a configuration, some modification needed to be introduced in the configurational nomenclature. Figure 2-6 exemplifies the configuration nomenclature applied to a typical compound with an asymmetric atom.

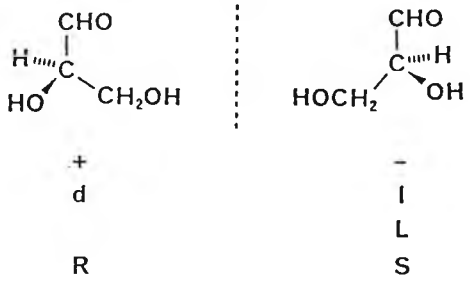
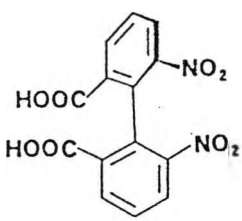


Figure 2-6

To assign the configurational term R or S, the biphenyl must be viewed in the conformation where both benzene rings are perpendicular to each other along the axis of the interannular bond. The four relevant o-substituents are projected on a plane at right angles to the axis, and the configurational term R or S is assigned as if the projection represented an asymmetric atom. If the substituents are not all different, a new rule 'the proximity rule' can be applied: The substituents on the ring parallel to the page precede the substituents on the perpendicular ring. Examples are given in Scheme 2-8. The configuration symbol is (R) because groups 1, 2 and 3 describe a right hand turn, (S) a left hand turn.



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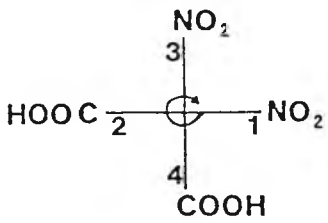

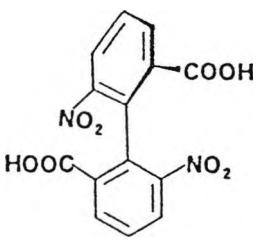

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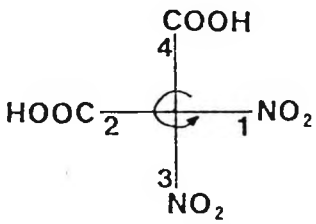
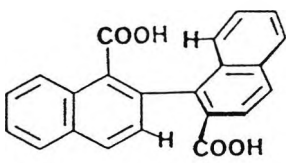


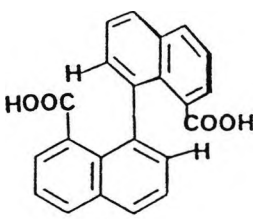
Scheme 2-8

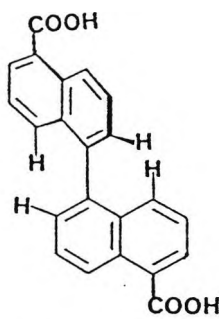
Binaphthyl molecules are also optically active atropisomers following the rules of restricted rotation as studied for biphenyl molecules. In 1928, Kuhu and Albrech resolved 2,2′ binaphthyl-1,1′-dicarboxylic acid (22) and found the enantiomers to be optically stable.

In 1931 Stainley resolved 1,1′-binaphthyl-8,8′--dicarboxylic acid (23) by asymmetric transformation using brucine .

In 1951 Bell and coworkers resolved 1.1′-binaphthyl 5,5′dicarboxylic acid (24), where the free rotation is impeded only by hydrogen atoms.

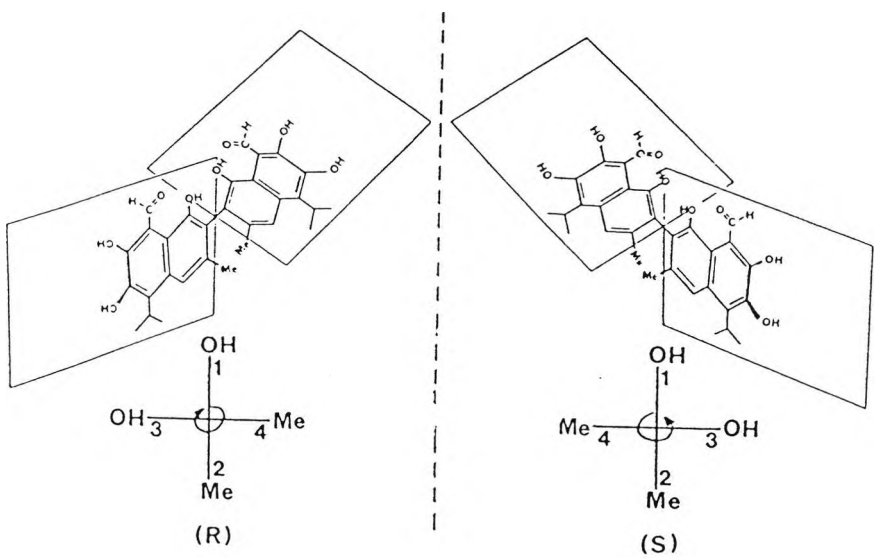






(22) (23) (24)

In the gossypol molecule the two naphthalene rings cannot be coplanar due to impingement of the o-methyl and o-hydroxy substituents (Scheme 2-7). The lack of planarity of the nuclei originates dissymmetry in the molecule.



Scheme 2-7.

From the cotton plant only racemic gossypol was isolated, usually as an acetic acid complex. In 1968, King and de Silva[69] isolated (+)-gossypol as the excess enantiomer from Thespesia populnea, with an optical rotation $[\alpha]^{19}$ = +445±10°. Prior to the present work, excess (-)-gossypol had not been isolated from any plant extract. In 1984 Matlin and Zhou[100] resolved chromatographically (-)-gossypol by conversion to diastereoisomeric Schiff's base derivatives using a chiral amine, (+)-1-phenyl-ethylamine. An optical rotation $[\alpha]^{26}$ = -363.6 (c=0.20 in MeOH) was found.

In 1988, Zhou and Lin[101] published the first report of excess (-)-gossypol occurring naturally and the isolation of enriched (-)-gossypol isomer from cotton seed plant, specifically from G. barbadense L.

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CHAPTER 3

RESOLUTION OF RACEMIC GOSSYPOL

3.1 Methods of Resolution of Racemates

Optical isomers occur widely in nature and are utilized for pharmaceutical purposes, for experimental biochemical studies, and as intermediates in the synthesis of biologically active products. Optical activity is an innate property of life and of most chemical materials of biological origin. When an asymmetric structure is synthesized by living media, almost always only one of the two mirror image isomers results. Chemical synthesis involving an asymmetric carbon structure often produces a racemic mixture of enantiomers, but usually only one form can be used for biological purposes. The importance of methods which distinguish or resolve enantiomers is great, whether it is to supply pure enantiomer or to assess the optical purity of a chiral compound.

A pair of enantiomers or asymmetric isomers will rotate the plane of polarised light by equal amounts in opposite directions; the prefixes d- and l- refer to right hand and left hand rotation. Therefore the conventional method to measure optical purity utilized a polarimeter. The accuracy of this method is dependent on the magnitude of specific rotation, on chemical purity and on the presence of any other optically active molecules. It also needs relatively large amounts of sample. An alternative quantitative method involving the separation of both enantiomers, free of possible impurities present, would be preferred.

The separation or resolution of two enantiomers in the absence of an optically active medium is not possible since all the physical properties will be the same. The reaction of a pair of enantiomers with optically inactive compound will form a pair of compounds at equal reaction rate constant, which still presents equal physical properties. Only when a pair of enantiomers reacts with an optically active compound, the diastereomeric products (non mirror image compounds containing two or more asymmetric centres) possess different physical properties and consequently resolution can be achieved.

Isomers have been resolved by direct or indirect methods. A direct resolution of enantiomers usually means the physical separation of such isomers without the formation of separate, distinct diastereoisomers by the use of an optically active resolving agent. Indirect resolution involves the use of such diastereoisomers.

In 1848, Pasteur performed the first physical resolution of a racemic mixture. Pasteur allowed the sodium ammonium salt of tartaric acid to crystallize by slow evaporation of a dilute aqueous solution. He observed that the crystals formed possessed a characteristic facet, which could be visually recognized as belonging to 2 groups. The characteristic facet from one group of crystals pointed in one direction, those of the other group in the opposite. Pasteur separated the crystals mechanically, with a pair of tweezers, into the two distinct groups, which later proved to be separate enantiomers. Later studies by other scientists demonstrated that slow crystallization of the sodium ammonium salt of tartaric acid carried out above 27°, would have led to a racemic compound, where mechanical separation is impossible.

This tedious method of mechanical separation can only be applied to racemic mixtures where the enantiomorphous crystals can be visually classified into two distinct groups.

In 1866, Gernez developed a more practical technique of direct separation of the enantiomers in a racemic mixture. This technique consists of seeding a saturated solution of a racemic mixture with crystals of the selected enantiomer. A supersaturated solution of the selected enantiomer is now formed, in which some of this compound begins to crystallize preferentially on cooling. It has been observed that preferential crystallization can also be achieved when a saturated solution of racemic mixture such as tartrate is seeded with crystals of a different optically active isomorphous molecule, such as (-)-asparagine, inducing (+)-tartrate to crystallize preferentially.

In a few cases only, seeding with crystals of a symmetrical molecule can lead to preferential crystallization of one enantiomer. This is the case when a solution of racemic asparagine is seeded with crystals of glycine.

Several spontaneous crystallizations of enantiomers from racemic mixtures have been reported.[1] Crystallization is a technique often used to purify partially resolved enantiomeric substances.

In 1957 Klingmueller and Gedenk described a physical separation of enantiomeric mixtures by dialysis. They partially resolved tartaric acid by dialysis through an optically active membrane.

Pasteur devised another resolution method for optically active compounds with an acid or alcohol group in their formula. He formed the ester of the compound with a levo or dextro isomer of some optically active alcohol (or acid). He

found that the two resulting esters, no longer enantiomers but now diastereomers, had different physical properties (e.g. solubility or boiling point) making separation possible by physical methods such as fractional crystallization. This resolution method was named chemical resolution.

The resolution of racemic mixtures by the formation of diastereomeric derivatives is not limited to compounds containing acid or alcohol groups. Any functional group of the racemic compound which reacts readily with an optically active molecule giving a derivative is suitable. The original enantiomers can be recovered by hydrolysis from the resolved diastereomeric derivatives.

Not all the diastereomeric derivatives are easily crystallized. The resolution of some racemic mixtures demands the examination of many resolving agents and the work on several different solvents to achieve crystallization.

The difficulty of resolution can increase by the formation of double salts, or when the solubility of the pair of diastereomers is quite similar.

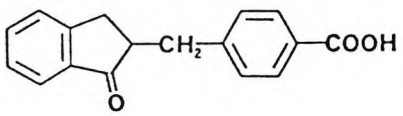
In general terms, the less soluble diastereomer crystallizes first quite pure, the other diastereomer may remain in an impure state, which can be purified by changing the resolving agent and even the solvent.

Most resolving agents are natural products and their enantiomers are not easily available.

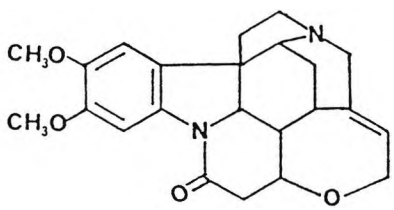
Although the expected resolution yields can be theoretically calculated to be no more than 50% from the racemic mixtures, cases were reported as high as 100%. This resolution was referred as second order asymmetric transformation and was only observed in optically labile

compounds. The occurrence was explained by the preferential crystallization of one diastereomer.

In 1921 Leuchs claimed to have obtained over 90% resolution when he reacted a racemic mixture of an indanone derivative (1) with brucine (2) in acetone, and also a racemic quinolone derivative with quinidine in methanol. In both cases epimerization had taken place by enolization. [1]

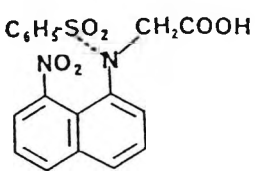


(1)



(2)

Similar examples were published with atropisomeric enantiomers, such as N-benzenesulphonyl-8-nitro-1-naphthyl -glycine (3), which presented restricted rotation around the C-N single bond. This molecule performs second order transformations in both directions, obtaining 98% of the (-)enantiomer derivative when treated with brucine (2) in acetone solution and 75% of the (+)enantiomer derivative when treated with brucine in methanol. [1]



(3)

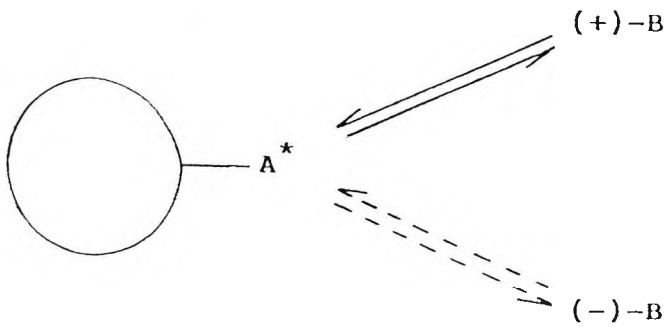
The traditional method of recrystallization of diastereomeric salts to resolve racemic mixtures is relatively difficult, inefficient and limited in its

applicability. Interest in research into alternative resolution techniques, mainly chromatographic techniques, has been greatly increased.

The chromatographic resolution of an enantiomeric pair has been done mainly by two general methods:

1. The most general but time consuming method is the initial preparation of a suitable diastereomeric derivative of the enantiomeric pair, followed by resolution on an achiral column packing, such as silica or ODS-silica, with mixtures of achiral solvents. Once the diastereomers have been resolved, the pure enantiomers must be liberated from the resolving agent by an appropriate hydrolysis procedure.
2. Direct resolution of enantiomers by stereospecific sorption on an asymmetric column packing.

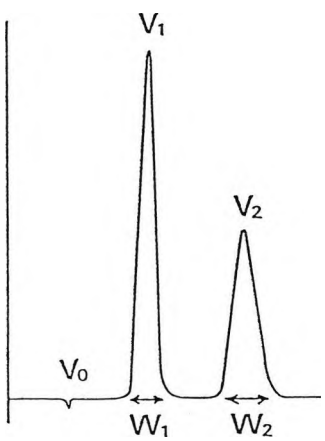
   This approach is more elegant and also analytically more sound, as the enantiomeric pair forms a diastereomeric complex with a chiral sorbent or solvent by solute sorbent (or solvent) interaction not involving the formation of a covalent bond (Figure 3-1). Here the relative stability of the pair of diastereomeric complexes is the controlling separation factor.



In situ formation of diastereoisomeric complexes

Fig. 3-1: Enantioselective interaction in chromatography

In assessing the performance of a column in liquid (or gas) chromatography, it is customary to use three standard parameters which describe the degree of retention and peak separation. The capacity factor k′ expresses the degree of retention of a peak as multiples of the elution time (or volume) of an unretained solute. The relative retention of two peaks, or separation factor α, can then be expressed as the ratio of their k′ values. However, this does not take account of the widths of the peaks and their possible degree of overlap at the baseline. Consequently, a more complete assessment of resolution R_s, expresses the separation both in terms of peak retention and average peak base width (Fig. 3-2).



$$k = \frac{V - V_0}{V_0}$$

$$\alpha = \frac{k'_2}{k'_1}$$

$$R_S = \frac{2(V_2 - V_1)}{W_2 + W_1}$$

Figure 3-2

Since the 1950′s, considerable research has been conducted into finding the right asymmetric column packing to resolve enantiomers directly by sorption. A number of interesting literature reviews have been published.[2-11]

Enantiomer-specifically double-labelled racemates have proved very useful in the search for new adsorbents. Mandelic acid was at first used as a racemate for testing the efficiency of column resolution, one enantiomer being labelled with ^{3}H and the other with ^{14}C. The concentration of one enantiomer is accordingly proportional to the ^{3}H and that of the other to the ^{14}C activity. Since no impurities interfere with radioactivity measurements, double labelling makes it possible to determine the resolving power of new adsorbents rapidly and reliably.

Stereospecific sorption of enantiomers on asymmetrical packing materials has been described by the model of the 'three points of contact'. Since enantiomers differ only in their 3-dimensional structure, the position of a minimum of 3 groups must be identified to distinguish between members of an enantiomeric pair. It was Ogston[12] in 1948 who first recognized the importance of the 'three points of contact' theory for stereospecific enzyme reactions. Later this theory has been widely used to explain the resolution of racemates by asymmetric sorbents and by optically active solvents. The 3 points of contact between sorbent and solute are mainly achieved by H-bonds, electrostatic and complexation interaction, although some scientists believe that steric interaction can count as one of the three.

Proteins

Natural proteins constituted the first disymmetric sorbent used in studying the stereoselective sorption of enantiomers. Initially it was attempted to resolve the enantiomers of racemic azo-dyes on the proteins of wools with little success. A more detailed study of the sorption of α-hydroxy acids on wool was made by Bradley and his

coworkers[5,13], who reached the conclusion that the interaction points were mainly through the free basic groups of L-lysine and L-arginine in wool. This observation was supported by the lack of resolving power of wool whose basic groups had been previously blocked by the radicals of some other acids. The inability of silk, which contains only minute quantities of diamino-carboxylic acids, to sorb selectively the enantiomers of racemic acids is also significant. By studying the separation on wool of the isomers of a large number of racemic α-substituted glycol ic acids, Bradley[14] showed that large substituents promote non selective adsorption, decreasing with the size of the substituent the degree of resolution. These facts make clear the reason for failure of earlier attempts to resolve large molecules of azo-dyes.

Carbohydrates

Natural carbohydrates such as lactose, cellulose, starch and sucrose have also been used in the resolution of racemates. Carbohydrates contain no functional group capable of electrolytic dissociation in neutral media, and therefore the resolution of racemates has to be achieved by other intermolecular forces, mainly hydrogen bonds. A success in this field was the resolution of Troeger's base on a lactose column in 1944 by Prelog and Wieland.[15] This compound could not be resolved by other methods owing to its instability in acid media. During the 1950's considerable progress was made in the resolution of racemates by chromatography on cellulose (paper). As early as 1948 Dent[16] described the unexpected splitting of chromatographic spots of aspartic and glutamic acids on cellulose paper.

In 1952 Dagliesh[17] reported that the resolution of racemates on cellulose paper did not involve partition

chromatography as had previously been assumed, but represented a method of adsorption chromatography. Hydrogen bonds play an important role in the process of adsorption. Dagliesh did extensive work on the resolution of amino acids and concluded that for successful resolution of the racemate of an amino acid, its molecule should contain a free carboxyl and a free amino group. A planar aromatic ring having a small ortho substituent would provide a third functional group to ensure three point adsorption. Dagliesh's rules concerning three point sorption are only approximate, and it was proved by others that the presence of an aromatic group is not essential for resolution on paper.

Similar to cellulose are the crosslinked dextran materials (polyglucose) sold under the trade name of Sephadex. Only partial resolution of enantiomers, like mandelic acid, was achieved.[18]

Krebs et al[19] succeeded in his work with starch, as a natural disymmetric sorbent suitable for the resolution of racemates. Starch has proved capable of partly resolving a large number of racemates consisting of complex salts of cobalt and chromium containing two or three bidentate ligands such as glycine, dithiocarbamic acid, ethylendiamine, oxalic acid, etc. The resolution of these complexes was more effective on starch than on lactose, cellulose, sucrose or wool. Krebs considered the use of Dagliesh's rule on three point adsorption only valid for flexible aliphatic molecules. Rigid molecules like phenylglycine would only need two or even one functional group capable of forming hydrogen bonds with the hydroxyls of the starch, to be sufficient for successful resolution.

Several carbohydrates sorbents are commercially available under the trade name of Chiralcel (Figure 3-3).

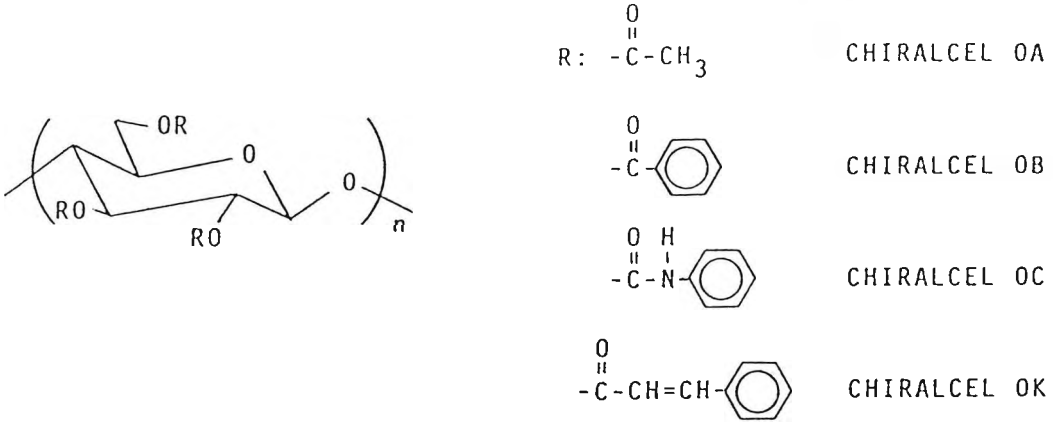


Fig. 3-3: Chiral sugar sorbents

Organic and inorganic disymmetrical sorbents

Amongst inorganic disymmetrical sorbents, finely ground crystals of optically active quartz have been used for resolving racemates. A partial separation has been effected of the octahedral complex salts of cobalt(III) and chromium(III) with two or three bidentate ligands, amongst them ethylenediamine, dimethyl-glyoxime, oxalate.

Resolution of racemates on the organic and inorganic disymmetrical sorbents described above are only partial. Complete resolution was achieved on microcrystalline non-ionic cellulose. Wool has never been used for chromatographic type resolution as the packing density for wool is low and the kinetics of sorption are very poor due to wool's heterogeneous nature. Although chromatography on cellulose can be conducted on a preparative scale in columns, the degree of separation of the enantiomers then drops sharply.

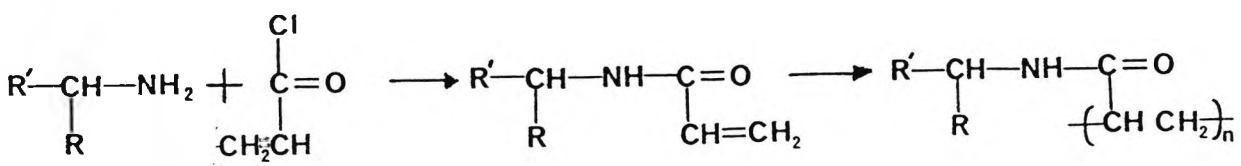
Asymmetric synthetic resins and polymers

To improve the resolution of the enantiomeric pair on the sorbent, different resins were synthesized as asymmetric sorbents.

Burnett and Mark[20] in 1952 synthesized a condensation polymer of the phenol-formaldehyde type, where the phenol group was part of an asymmetric molecule like tyrosine. No resolution was achieved.

In 1967 Nakamura et al[21] incorporated α-amino acid derivatives into vinyl-type polymers and reported partial resolution of dl-mandelic acid.

A great advance was made by Blaschke[6] in the synthesis of optically active polymeric supports, prepared by polymerization of optically active olefinic amides acting as the monomers. A very large number of different optically active amines were prepared and reacted with various allylic acid chlorides to yield unsaturated amides. These amides were then polymerized under a wide variety of conditions to give various polymer types (Scheme 3-1).



Scheme 3-1

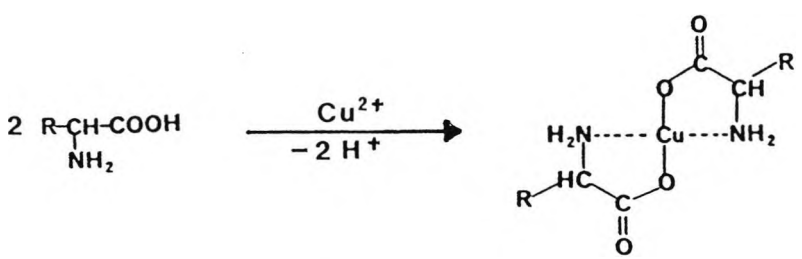
Some of these polymers were used successfully for the resolution of optically active mandelamine and mandelic acid, the latter acting as markers for the effectiveness of the resolution possible on each polymer. It was also possible to resolve other material partially, such as amides, amino acids, etc. Typical eluting solvents were benzene, dioxane and benzene/cyclohexane. For certain substances the resolution was very good, however for the majority of compounds studied the resolution was only partial.[6]

Although Blaschke's polymeric supports appeared to offer some degree of success, the resolving ability of the polymeric material depends on many factors such as the basic structure

of the monomer used, the polymerization process used, the initiating agent, solvent and temperature. The final nature of the optically active polymer formed depends on all these factors. The amount of crosslinking present must be varied in order to minimize the final swelling properties of the polymer. The swelling occurs with the nature of the solvent used as an eluent. Because of the crosslinking, the adsorbents are insoluble and mechanically stable, can be dried and swelled again and can be used for chromatography as often as desired without losing their resolving power. The resolution is thought not to be due to the resolving action of polyamides, more likely the resolution proceeds because of different fittings of the enantiomers into asymmetric cavities in the polymer chain.[6]

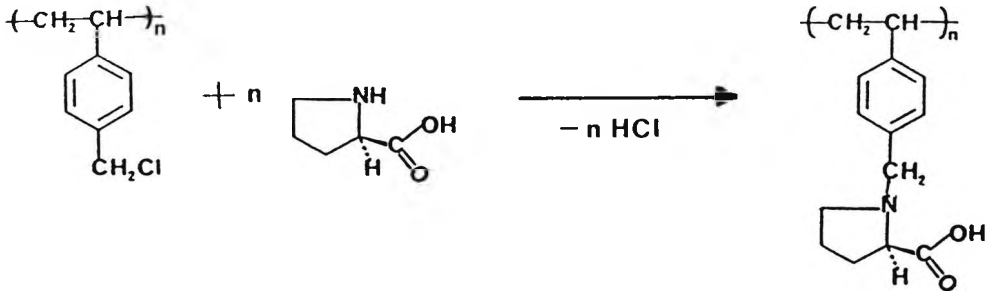
Chiral ligand-metal complexes

Ligand chromatography particularly has been developed by Davankov[7] for the resolution of racemic α-amino acids. A very suitable absorbent is polymer bound L-proline. Amino acids in aqueous solution form complexes with Cu^{2+} ions, in which the central atom is bonded to two amino acid molecules (Scheme 3-2).



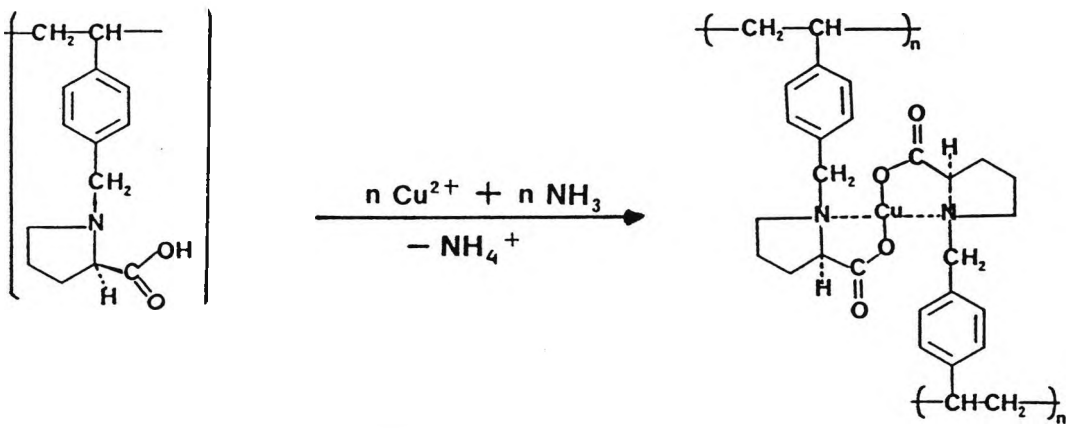
Scheme 3-2.

In ligand chromatography the adsorbent is formed first by bonding covalently an optically active ligand (amino acid, in this example L-proline) to an insoluble carrier (chloromethylated polystyrene) (Scheme 3-3).[7]



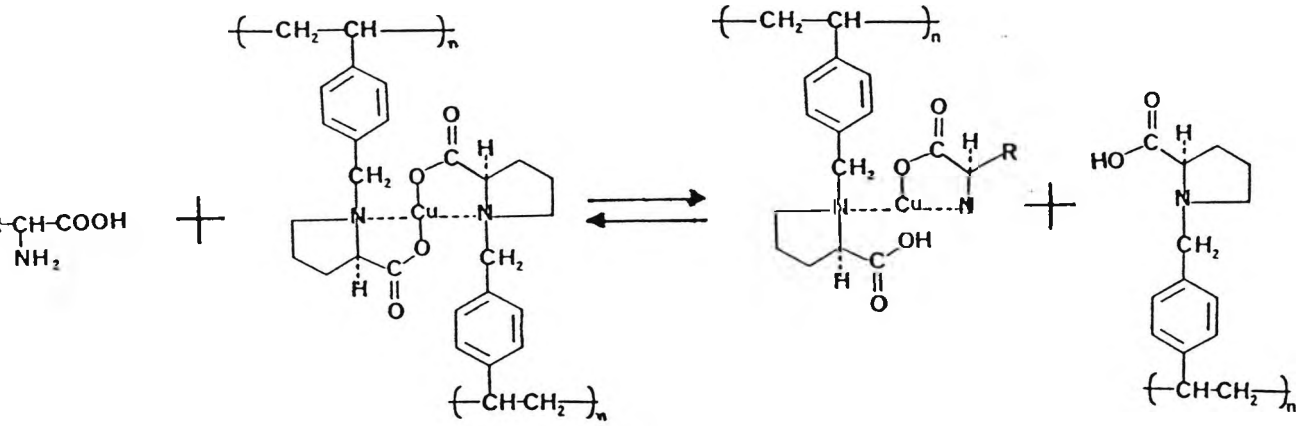
Scheme 3-3

This adsorbent is then charged with Cu^{2+}, by treating it with ammoniacal copper(II) nitrate solution, forming 2:1 adsorbent Cu^{2+} chelate complex (Scheme 3-4).



Scheme 3-4

The enantiomers, such as α-amino acids, then form diastereomeric complexes with Cu^{2+} ions and the adsorbent. The differences in complex stability enable chromatographic resolution (Scheme 3-5).[7]

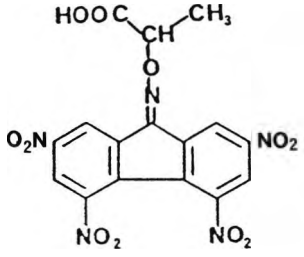


Scheme 3-5

Racemic proline can be resolved especially well on the proline polymer. Many other amino acids can be partially or completely resolved into enantiomers on the L-proline polymer/Cu^{2+} complex adsorbent.[22] Resolution is lower when other adsorbents are prepared by bonding polystyrene to α-amino acids such as L-hydroxy-proline, L-allohydroxy proline and complexing with Ni^{2+} ions.[23]

Chiral charge-transfer complexes (Pirkle phases)

In the late 1970's Gil-Av et al[24,25] developed new chiral packing materials whose resolution principles were based on the formation of chiral charge-transfer complexes with the solute. Gil-Av first linked optically active charge transfer acceptors such as α-(2,4,5,7-tetranitro-9-fluorenylidene-aminoxy) propionic acid "TAPA" (4) and similar acceptors to silica gel by adsorption and then resolved enantiomers such as helicenes (5) in solvents in which TAPA is insoluble.



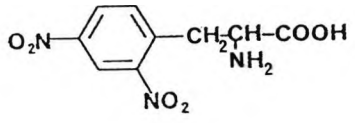
TAPA (4)

helicine (5)

These optically active resolving agents form charge transfer complexes with both enantiomers of the helicenes. Since these charge transfer complexes are really diastereomeric complexes, they will have different equilibrium constants for their formation and dissociation on the silica gel support. Low temperature (less than 0°C) improved resolution.[24,25]

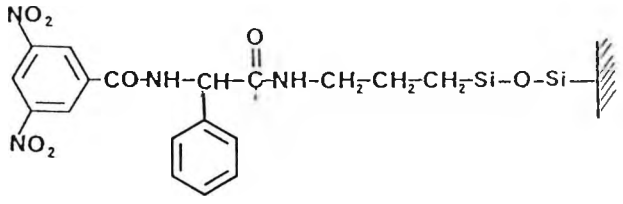
A further development by Gil-Av was to directly attach by covalent bonding a number of chiral charge transfer complexing agents to the silica gel supports, and subsequently directly resolve a number of helicenes on these columns. Gil-Av bonded his optically active charge transfer materials to the silica gel via a linkage molecule of 3-aminopropyl triethoxy-silane. This step was followed by the attachment of the chiral carboxylic acid [e.g. R(-)-TAPA] to the aminated silica gel using an appropriate coupling reagent. Amongst his chiral charge transfer reagents are TABA, which is the 2-butyric acid analog of TAPA.[24,25]

Lochmueller[8] also worked with chiral charge transfer packing materials, bonding the 2,4-dinitrophenyl alanine (6) to silica to resolve hexahelicenes.



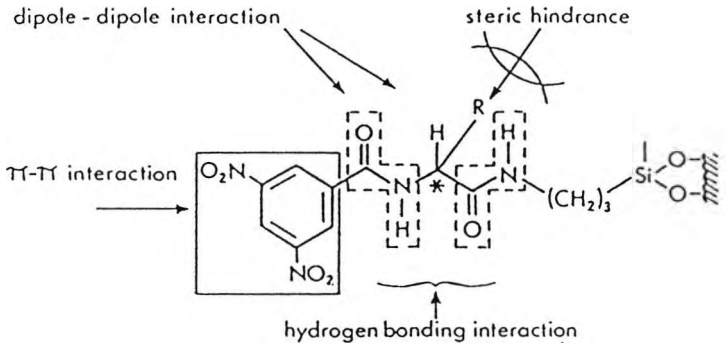
(6)

Pirkle[9], in 1981, synthesized a series of chiral charge transfer molecules such as (R)-N-(3,5 dinitrobenzoyl) phenyl glycine and bonded it to an aminopropyl silica packing material (7).



(7)

A wide range of racemates of naphthalene, anthracene, and other polynuclear aromatic derivatives were successfully and completely separated by this technique, using isopropanol (i-PrOH) in hexane as mobile phase.[9] The possible sites of interaction on a Pirkle type chiral stationary phase is represented in Fig. 3-4.

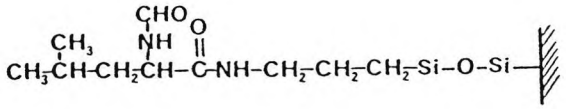


Possible stereoselective sites of interaction on a Pirkle-type CSP; the dashed lines mark out amide dipoles.

Fig. 3-4: Pirkle type and related stationary phases.

Hara and Dobashi[26] bonded N-formylvaline to aminopropyl silica and successfully resolved N-acylaminoacid esters.

Following this, Akanya et al[10] bonded N-formyl--L-leucine (8), N-formyl-L-isoleucine (9), N-formyl-L-phenyl alanine (10) and N-formyl-L-proline (11) on aminopropylsilica packing and resolved partially or totally a range of amino acid derivatives and related racemates using i-PrOH/hexane as mobile phase. Akanya observed that the t-butyl-ester derivatives resolved best, therefore indicating a steric element in the chiral interaction, optimized with a bulky alkyl group with branching close to the chiral centre. Since it was observed that resolution deteriorated rapidly with increase in isopropanol concentration, one of the other interactions must be ascribed to hydrogen bonding.



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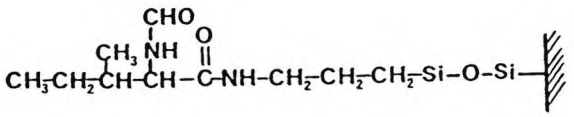

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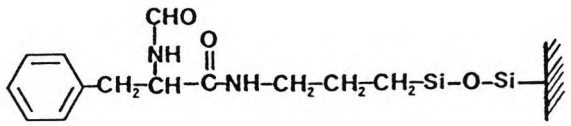

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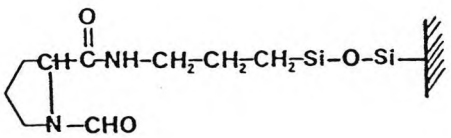
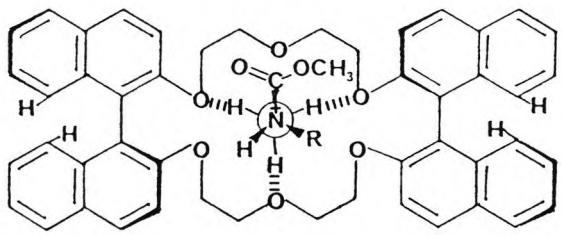


(11)

Chiral crown ether

Chiral crown ether sorbents were developed by Cram[27] showing strong resolution power for racemic alkylammonium ions or amino-alkyl compounds. Crown ethers form complexes not only with alkali metal ions and alkaline earth metals but also with amines or ammonium groups (12). The hydrogen atoms of the ammonium group are hydrogen bonded to the oxygen of the crown ether.



(12)

Cram and his coworkers have developed optically active crown ethers such as the chiral binaphthyl derivative, which are chiral due to restricted rotation around the binaphthyl axis. This stationary phase has been utilized for racemate resolution of alkylammonium ions of structure $R\text{-}NH_3^+$, in which chiral groups R are drawn into the asymmetric cavity of the crown ether and therefore form diastereomeric complexes with various degrees of stability (12). To perform a direct LC resolution, Cram attached the chiral binaphthyl crown ether molecule directly to the silica gel, through only one Si-O-Si bond to the naphthyl ring. The other 3 reactive functions of the other 3 naphthyl groups were endcapped with $Si(CH_3)_2OCH_3$ groups to prevent any tailing of the solutes during the elution process. Salts of p-hydroxyphenylglycine, phenylalanine, tyrosine and tryptophan have been resolved completely on silica gel substituted with a derivative of crown ether with separation factors up to 6.4. In order to obtain separation on this phase it is absolutely necessary that the chirality centre of the solute must be directly adjacent to the ammonium group.[27]

Cram obtained an even better resolution when the optically active crown ether was bonded on a cross linked polystyrene material. With an adsorbent of this kind, there is no need of endcapping and it can stand enough back pressure to be used also in HPLC. It is suitable for the resolution of racemic α-amino acids and their methyl esters as perchlorates, obtaining separation factors as high as 20. Unfortunately, such adsorbents are very difficult to prepare.[27]

Cyclodextrins

Cyclodextrins are torous-shaped cyclic oligosaccharides which are composed of α-1,4 lined glucopyranose units. The cyclodextrins, which exist in α-, β- and γ-forms depending on the number of interconnected glucose units (6, 7 or 8, respectively), exists as levorotatory helices. Each cyclodextrin structure has different properties, determined by the particular arragement of its functional groups. The structure of β-cyclodextrin is shown in Fig. 3-5.

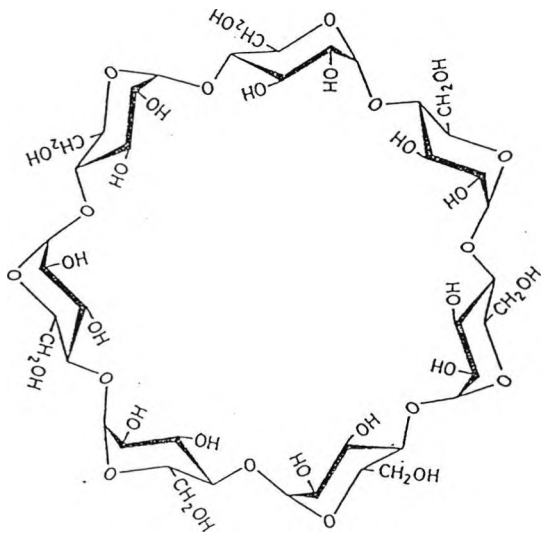
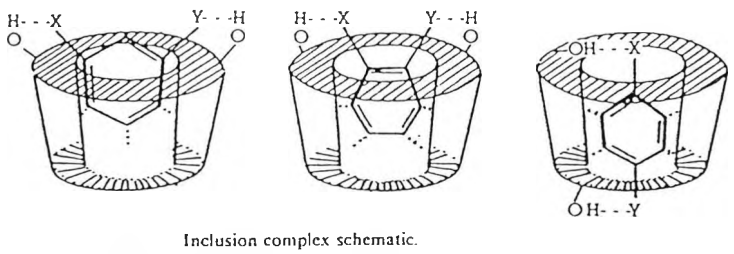


Figure 3-5: Structure of b-cyclodextrin

Early work[29] appeared to indicate that β-cyclodextrin could form inclusion complexes with various organic compounds of acidic, neutral and basic character, as illustrated in Fig. 3-6.



Inclusion complex schematic.
--- Hydrogen bonding
... Electrostatic association
X Hydrophobic groups that can enter cavity
Y Polar groups that can hydrogen bond.

Figure 3-6 Possible orientation of guest molecules in a cyclodextrin host.

Table 3-1 tabulates the cavity diameter of different cyclodextrins, sold commercially under the trade name of Cyclobond.

Table 3.1 Cyclobond columns

Name	Type	Cavity diameter Å
Cyclobond I	β-cyclodextrin	7.5
Cyclobond II	γ-cyclodextrin	9.5
Cyclobond III	α-cyclodextrin	5.7
Cyclobond I acetylated	β-acetylated	7.5
Cyclobond III acetylated	α-acetylated	5.7

3.2 Analysis and Resolution of Gossypol : Literature Survey

Throughout the initial toxicity and antifertility studies of gossypol, the analytical methods used to quantify gossypol were mainly gravimetric, titrimetric and spectrophotometric. These methods are not specific since gossypol and other related pigments may react similarly to the same analytical reagents. The gravimetric and colorimetric analysis depends on the reaction of aniline with the two carbonyl groups of gossypol. Carbonyl groups of degradation products present may also react, therefore interfering with the analysis.

To overcome this problem, analyses of gossypol utilising chromatographic techniques including paper, thin layer and gas liquid chromatography have been developed. However these methods are time consuming, lack in sensitivity or require prior derivatization.

From 1980 HPLC methods were developed to analyse gossypol content in plant extracts and to assess gossypol stability in different solvents. Reverse phase HPLC on an ODS silica column using a methanol/water mobile phase achieved nanogram detection limits compared to microgram detection limits previously obtained by spectrophotometric methods. Within a 10 minutes run, gossypol was separated from the degradation products. Addition of 0.1% phosphoric acid to the mobile phase succeeded in suppressing the ionization of the phenolic hydroxyl groups of gossypol, therefore avoiding tailing of the gossypol peak.[28,29]

Jefford et al[30] claimed to improve on the previous method by using acetonitrile, obtaining sharper peaks at similar retention time. Jefford et al also observed that addition of a salt such as tri-n-butylammonium phosphate

adjusted to the pH 3.5 with phosphoric acid, provided a peak for gossypol of excellent symmetry. A further reason for the avoidance of methanol in the eluent is the instability of gossypol-acetic acid in methanol, while in acetonitrile it is stable for over 2 weeks.

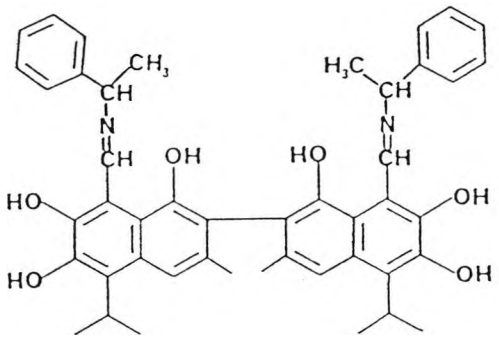
During the 1980's a need to determine the presence of gossypol in plasma was appreciated by various scientists. Tang et al[31] detected plasma gossypol using a radioactive labelling method. However this method can not really distinguish between gossypol and its metabolites. Various HPLC methods were subsequently developed for the quantitative and qualitative analysis of plasma gossypol.

Sattayasai et al[32] extracted gossypol from plasma by precipitating the proteins with absolute ethanol and added the chelating agent EDTA to break down existing gossypol metal complexes into free gossypol. Gossypol was then extracted with benzene, evaporated and redissolved in methanol. They obtained a good separation on an ODS column with a methanol/water/acetic acid mobile phase monitoring at 254 nm.

Wang et al[33] used a column switching technique on a reverse phase HPLC method monitored with an electrochemical detector. The acetonitrile-treated, protein-free plasma sample was concentrated and cleaned up on a C-18 precolumn and later separated on a C-8 column using a methanol/citrate buffer mobile phase. Glutathione was used as a protecting agent at the stage of sampling and gossypol dimethyl ether was chosen as an internal standard. Recovery was as high as 95% and the detection limit in plasma was 5 ng/ml. This method was found to be particularly suitable for clinical pharmacokinetic studies of gossypol.

All the clinical work carried out in China with gossypol as an antifertility agent had been done with the racemic form. Following the isolation of (+) gossypol in 1968 by King and de Silva[34] from Thespesia populnea, subsequent animal testing showed that it was inactive in rats and hamsters as a male oral antifertility agent. This finding, around 1980, implied that (-)-gossypol had to be the active enantiomer, leading to the search for a source of the pure (-)-gossypol enantiomer. At this time, no plant had been found containing an excess of (-)-gossypol and consequently an investigation of methods for the resolution of racemic gossypol was initiated.

In 1984, in the City University, Matlin et al[35-38] developed a method for the resolution of gossypol and achieved the preparation of 2g of pure (-)-gossypol. Initially an attempt was made to resolve gossypol by the classical procedure of fractional recrystallization of diastereomeric derivatives. Gossypol was converted into Schiff's bases by condensation with chiral primary amines such as 1-phenylethylamine (13) and phenylglycine ethyl ester (14), but no resolution was observed on recrystallization.



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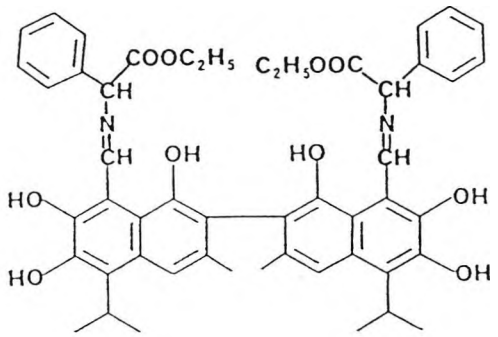
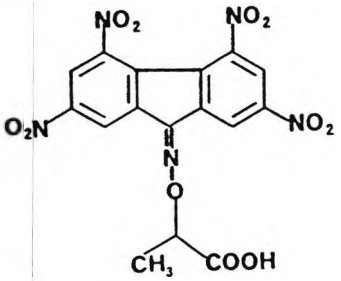
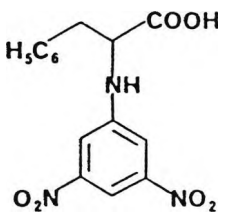
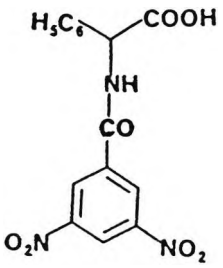


(14)

An alternative chromatographic separation by HPLC was therefore investigated. A number of chiral columns were prepared by reaction of aminopropyl silica with optically pure amino acid derivatives, such as (-)-α-(2,4,5,7-tetranitro-9-fluorenylidene aminoxy) propionic acid (-)-TAPA) (15); N-3,5-dinitrophenyl-1-(+)-phenylalanine (16), and N-(3,5-dinitrobenzoyl)-D-(-) phenylglycine (17).







(15) (16) (17)

Salts were formed by mixing the reactants in THF, and the amide form of TAPA was prepared by a carbodiimide coupling procedure for obtaining a stationary phase which is stable in more polar mobile phases.

Initial attempts by Matlin and Zhou[35-38] to resolve gossypol directly on these chiral columns were unsuccessful. Therefore separation of the Schiff's base diastereoisomers of gossypol was attempted on the chiral phases. Although (+)-phenylethylamine gossypol Schiff's base diastereomers had previously failed to resolve by fractional recrystallization, they succeeded in being resolved on all chiral phases, the diastereoisomer derived from (-) gossypol always being eluted first. The best resolution was obtained with an α of 1.33 on the chiral phase containing the phenylglycine derivative, using 90:5:3:2 hexane/CH_2Cl_2/MeCN/ i-PrOH. The performance of the amide form of(-)TAPA column was slightly better than that

of the salt form. The phenylglycine derivative column was finally chosen from the point of view of economy and ease of synthesis.

This resolution established a suitable HPLC method for the evaluation of the optical purity of gossypol samples: 1 mg or less of racemic gossypol was converted to the Schiff's base of (+)-phenylethylamine after stirring in isopropanol for 1 hour, then it was evaporated to dryness and redissolved in dichloromethane for HPLC.[36]

By scaling up the chiral column to a 2.5 cm o.d. preparative column on a 5 μm silica bonded packing, gram quantities of (-)-gossypol diastereomer was obtained. The capacity of the chiral column was 120 mg of gossypol derivative per injection with baseline resolution.

The (-)-gossypol enantiomer was recovered from the derivatives by alkaline hydrolysis (two successive treatments with MeCN/aq. KOH).

Subsequently, Matlin and Zhou[36] found that the use of a chiral amine to form the Schiff's base is not essential and good resolution on the chiral columns were obtained with imines from simple amines, such as propylamine and aniline.

In 1985 L. Huang et al[39] from the Institute of Materia Medica in Beijing, China, also published a method of resolution of gossypol. They accomplished the resolution by chromatographic separation of the (S)-1-methylphenethylamine Schiff's base of racemic gossypol hexa-acetate (Figure 3-7) on a silica column with diethyl ether/light petroleum ether as eluant. The (-)-diastereomer eluted first. Pure gossypol enantiomers were obtained by acid hydrolysis followed by recrystallization.

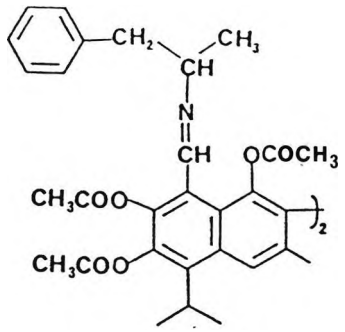


Figure 3-7

3.3 Objectives

The varied activity of the enantiomers of gossypol, and especially the potent anti-spermatogenic and anti-tumour properties of the (-)-isomer, have led to a requirement for large quantities for further biological studies. The main objective of this study was the production of 40 g of the (-)-isomer and over 20 g of the (+)-isomer of chemically and optically pure gossypol for toxicity tests on male bonnet monkeys. It was also intended to supply the world's scientists interested in the study of gossypol with 20mg samples of pure (+)- and (-)-gossypol to be used as standards. This work was commissioned by the WHO Task Force on Methods of Male Fertility Regulation, as part of a three centre collaborative programme, with L. Huang from China and Palmer Research Laboratories in Wales.

Our work was initiated on the understanding of a close collaboration with Palmer Research Laboratories, who were asked to carry out a large scale separation of the enantiomers of gossypol. Simultaneously, by independent methods, L. Huang was commissioned also to prepare gram quantities of chemically and optically pure (-)- and (+)-isomers of gossypol.

Since the methods so far reported by Matlin's group and others were unsuitable for large scale operation (because of poor resolution or because of the expense of constructing large columns with chiral bonded phases), we were asked to develop a new procedure suitable for preparing multigram quantities of the enantiomers.

The work was started by first looking for a suitable diastereoisomer of gossypol, which would to some degree resolve preferentially by fractional recrystallization, or if not then by a chromatographic technique.

The next step was to investigate a suitable method of hydrolysis of the gossypol enantiomer derivatives with the aim of obtaining both gossypol enantiomers with chemical and optical purity higher than 99%.

A study of purification by recrystalization of enriched enantiomeric crops was investigated to obtain enantiomers of high purity.

Various chromatographic methods were investigated to assess accurately the optical and chemical purity of gossypol samples. A preparative HPLC system was studied for the large scale resolution of gossypol.

Additionally we were asked to assess the optical and chemical purity of all the gossypol enantiomer samples produced in the multicentre study and to combine them into a final batch of gossypol with a chemical and optical purity of not less than 99.8%, and produce a micronized batch suitable for toxicity tests.

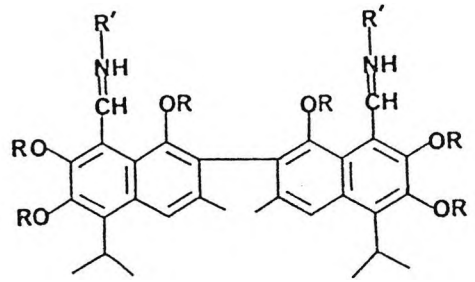
3.4 Preliminary Studies by Palmer Research Laboratories

Tyson and Brookes working for the Palmer Research Laboratories were commissioned by the WHO to research into a viable gram scale resolution of the enantiomers of gossypol.

The most suitable and fast large scale resolution technique would be by a classical procedure of fractional crystallization of diastereomers. Based on the lead generated by Matlin and Zhou's earlier work,[36] samples of gossypol Schiff's base derivatives of the chiral amines (18a)(Table 3-2) were prepared. The required Schiff's bases were successfully obtained using (+)-1-phenylethylamine, (+)-dehydroabietylamine, (+)-phenylalanine methyl ester and (-)-menthylhydrazide. Unsuccessful attempts were made to prepare derivatives of (+)-norephedrine and (-)-2-aminobutan--1-ol. Tyson and Brookes repeatedly crystallized the chiral amine derivatives (18a) (Table 3-2) from a number of organic solvents (alcohols, aromatics, halocarbons), but in each case there was no change observed in the optical rotation of the diastereomeric mixture. In view of the poor solubility of these Schiff's bases it was decided to prepare their corresponding hexaacetate derivatives (18b)(Table 3-3) to determine whether such compounds would more readily crystallize. The conversion of the four Schiff's bases to their corresponding hexa-acetates was not as readily accomplished as for gossypol itself. The purity of the compounds obtained was not high. The Schiff's bases with (+)-1-phenylethylamine and (+)-dehydroabiethylamine both gave two main hexa-acetates on exhaustive acetylation (Table 3-3), whereas the Schiff's bases with (+)-phenylalanine methyl

ester and with (-)-menthylhydrazine gave complex mixtures which were not studied further. Recrystallization of bases did not lead to any alteration in optical rotation.

The outline preparative details together with the physical properties of all the compounds isolated are shown in Tables 3-2 and 3-3.



a. R = H

b. R = $COCH_3$

(18)

Palmer Research subsequently directed their investigation into resolving the prepared chiral amines derivatives of gossypol in a chiral column by HPLC, following the lead from the investigation of Matlin and Zhou.[36]

Like Matlin and Zhou, Palmer Research found that some of their chiral amine derivatives of gossypol resolved well on a chiral bonded phase, such as N-3,5-dinitrobenzoyl--D-(-)-phenylglycine on aminopropylsilica using different mixtures of hexane: i-PrOH in the range 40:60 to 90:10 v/v. Of the 4 derivatives prepared, only those from (+)-1-phenylethylamine and (+)-dehydroabietylamine were resolved into two diastereomers on the chiral column (table 3-4). The study of the resolution by a chiral phase was also extended to non chiral amine derivatives of gossypol. All the prepared non chiral amines resolved into two diastereomers on the chiral column (Table 3-5). Details of their preparation and physical characteristics are listed in Table 3-6.

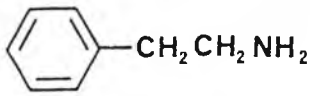
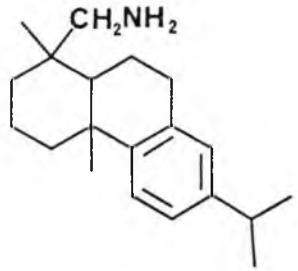
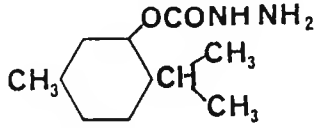
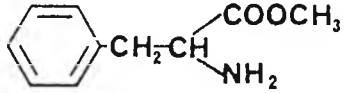
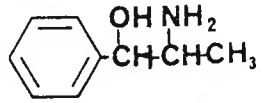
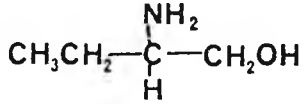
Gossypol derivative		m. p. °C	Reaction Solvent	Appearance	TLC silica (Hexane/EtOAc) (70:30)
(+)-1-Phenylethyl-amine		235-238	IPA	Yellow microcrystals	1 spot Rf = 0.44
(+)-Dehydroabietyl amine		165-168	IAA	Yellow-orange solid	1 spot Rf = 0.56
(-)-Menthyldrazine		196-199	IAA	Yellow solid	1 spot Rf = 0.68
(+)-Phenylalanine methyl ester		140-145	IPA	Yellow solid	2 spots Rf = 0.34 Rf = 0.26
(+)-Norephedrine		Could not be crystallized	IPA	-	
(-)-2-amino butan-1-ol		Could not be crystallized	IPA	-	3 spots in reaction solution

Table 3-2.
Gossypol Schiff's bases prepared from chiral amines

Table 3-3.
Gossypol hexa-acetate Schiff's bases prepared from chiral amines

Gossypol Derivative	m.p.	Reaction Solvent	Appearance	TLC silica (Hexane/EtOAc) (70:30)
(+)-1-Phenyl-ethylamine	160-163	Ac_2O/Py	Cream coloured solid	1 major spot Rf = 0.22 + minor impurities
(+)-Dehydro-abietylamine	157-165	Ac_2O/Py	Pale yellow solid	4 spots Rf = 0.60 to 0.70
(-)-Menthyl-drazine	158-163	Ac_2O/Py	White solid	5 spots Rf = 0.20 to 0.30
(+)-Phenyl-alanine methyl ester	155-160	Ac_2O/Py	Pale Yellow solid	5 spots Rf = 0.00 to 0.10

Table 3-4
HPLC separation of gossypol Schiff's bases prepared from chiral amines.

Amino component of Schiff's base	Mobile phase Hexane	IPA	k' Peak 1	Peak 2	α
(+)-1-Phenylethyl-amine	85	15	6.20	7.13	1.25
(+)-Dehydroabietyl-amine	85	15	2.20	3.17	1.44
(-)-Menthydrazine	80	20	5.87	-	1.00
(+)-Phenylalanine methyl ester	50	50	6.27	-	1.00

Table 3-5
HPLC separation of gossypol Schiff's bases prepared from non-chiral amines.

Amino component of Schiff's base	Mobile phase Hexane	IPA	k' Peak 1	Peak 2	α
Ethylamine	60	40	4.93	6.07	1.23
n-Propylamine	80	20	5.20	6.13	1.18
n-Butylamine	80	20	4.00	4.53	1.13
Iso-propylamine	80	20	4.33	5.13	1.18
2-Phenylethylamine	60	40	6.00	7.00	1.17
2-Thiophene-methylamine	60	40	9.27	11.60	1.25
3-Methoxypropyl-amine	60	40	4.47	5.27	1.18

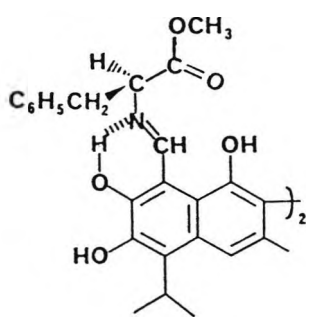
Table 3-6: Gossypol Schiff's base derivatives prepared from non-chiral amines.

PREPARATION		PHYSICAL PROPERTIES		
Gossypol derivative	Reaction solvent	Appearance	m.p. (°C)	TLC (Hexane/EtOAc 70:30 v/v)
Ethylamine Schiff's base	IPA/MeOH	Yellow solid	230-232	Single spot R_f = 0.23
Methylamine Schiff's base	IPA/MeOH	Yellow solid	230-235	Single spot R_f = 0.04
n-Propylamine Schiff's base	IPA	Yellow solid	245-250	Single spot R_f = 0.28
n-Butylamine Schiff's base	IPA	Yellow solid	205-210	Single spot R_f = 0.36
iso-Propylamine Schiff's base	IPA	Yellow solid	245-248	Single spot R_f = 0.26
Dimethylamino-propylamine Schiff's base	IAA	Yellow crystals	220-221	Single base-line spot
2-Phenylethyl-amine Schiff's base	IPA	Yellow solid	240-243	Single spot R_f = 0.40
4-Trifluoro-methylaniline Schiff's base	IAA	Orange solid	230-235	Single base-line spot
2-Thenylamine Schiff's base	IPA	Yellow solid	226-230	Single spot R_f = 0.36
3-Methoxy-propylamine Schiff's base	IPA	Yellow solid	220-224	Single spot R_f = 0.05

IPA = iso-propanol
IAA = iso-amylalcohol

The methylamine derivative was found to be too insoluble to inject. As the bulk of the amino substituent increases, solubility improves, but the resolution of the enantiomers becomes worse. As had been appreciated previously by Matlin et al, Palmer Research also realised that resolution on a multigram scale of gossypol via HPLC of a derivative on a chiral phase was not attractive.

From TLC studies on silica plates for the identification of the prepared amine derivatives of gossypol (Table 3-2) it was observed that the (+)-phenylalanine methyl ester Schiff's base of gossypol presented 2 similar intensity spots, both yellow in colour (Rf's 0.34 and 0.26 using hexane/ethyl acetate). The same gossypol Schiff's base of (+)-phenylalanine methyl ester had not resolved in a chiral HPLC column (Table 3-4). Although it was surprising to find that the diastereoisomers of the phenylalanine methyl ester Schiff's base of gossypol could not be readily separated by HPLC using the chiral column, it was more significant to find that the two isomers could be clearly separated using conventional silica gel chromatography. The TLC studies (Table 3-2) suggested that it would be feasible to separate the two diastereomeric components of gossypol by standard chromatographic techniques using a silica gel column.



(19)

The distinct difference in these two isomers may be explained by the intramolecular hydrogen bonding which is likely to be present in the molecule, as shown in (19). The rigidity of such a structure could give rise to distinct differences in the positions of the two phenyl groups of the phenylalanine moieties and this may account for the different physical properties of the two gossypol diastereoisomers. As a consequence, different affinities for silica gel would enable separation of the two components to be cleanly effected.

Palmer Research investigated the homologues of methanol used to prepare the (+)-phenylalanine methyl ester; initially the methyl, ethyl, isopropyl, t-butyl and benzyl esters of L-phenylalanine were obtained as the free ester from their hydrochloride or p-toluenesulphonate salts and reacted immediately with racemic gossypol to give the corresponding Schiff's bases. The isolated derivatives were then analysed by silica TLC to determine the difference between the polarities of the separated isomers (Details included in Table 3-7). It was found that on increasing the size of the ester group, the separation of the isomers on TLC became less distinct, with only one spot being observed for the t-butyl and benzyl esters.

A series of alkyl ester derivatives of other amino acids such as D-phenylglycine, L-leucine, and L-serine were also prepared and converted to Schiff's bases with racemic gossypol (Table 3-8). A TLC study on silica gave no better results than the derivative of (+)-phenylalanine methyl ester. The latter was therefore selected for scale up.

Table 3-7
TLC of racemic gossypol Schiff's bases with (+)-phenylalanine esters on silica.

Amino ester	Rf of product in hexane/EtOAc 70:30 v/v
L-Phenylalanine methyl ester	0.34 and 0.26
L-Phenylalanine ethyl ester	0.27 and 0.23
L-Phenlyalanine benzyl ester	0.5 (one spot only)

Table 3-8
TLC of racemic gossypol Schiff's bases with amino acid esters on silica.

Amino ester	Rf of product in hexane/EtOAc 70:30 v/v
D-(-)-p-Hydroxyphenyl glycine methyl ester	0.1
D-(-)-Phenylglycine methyl ester	0.26 and 0.21
L-Leucine methyl ester	0.32 and 0.25
L-Leucine isopropyl ester	0.5 (one spot only)
L-Tyrosine methyl ester	0.62 (one spot only)
L-Aspartic acid diethyl ester	0.1 (one spot only
O-Benzyl-L-serine methyl ester	0.1 (one spot only)

Large scale production of (+)-phenylalanine methyl ester Schiff's base of racemic gossypol (19) was accomplished by reaction of racemic gossypol with phenylalanine methyl ester in isopropanol with a yield of 85-90%.

Batches of 3-4 g. of material were eluted from a standard silica column (i.d 30 mm) with a 70:30 hexane/ethyl acetate mixture. In a typical run the enriched (-)-gossypol derivative was isolated first in a 50-60% recovery. The remaining material consisted of partially resolved (+)-gossypol compound, which could be recovered by elution of the column with ethyl acetate.

The resolved diastereomers obtained upon evaporation of the solvent were orange coloured oils. When attempts were made to purify the oil by crystallization from isopropanol, some racemization occurred. On trituration of the oil with hexane, a yellow powder was formed without any risk of racemization.

3.5 Results and discussion

3.5.1. Synthesis of gossypol Schiff's base with (+)-Phenylalanine methyl ester

The starting material was racemic gossypol acetic acid, supplied by Chinese scientists as a yellow crystalline solid. Preliminary solubility tests established that gossypol acetic acid was soluble in alcohols such as methanol and ethanol, in dimethylformamide (DMF), in aprotic solvent (ether, acetonitrile) but not in water. Gossypol acetic acid (m.p. 172-174°C) used as the starting material was chemically pure, as assessed by HPLC.

Before Schiff's base derivatives could be prepared, racemic gossypol had to be liberated from the acetic acid complex. This was accomplished by washing an ethereal gossypol acetic acid solution with water containing sodium dithionite (to prevent oxidation), followed by filtration of the solid product.

(+)-Phenylalanine methyl ester could be bought commercially only as the hydrochloride (HCl) salt. Before use, the associated HCl had to be removed. This was achieved by dissolving the HCl salt in dichloromethane and then extracting it with an aqueous solution of saturated sodium bicarbonate. The dichloromethane solution was then evaporated to dryness and quickly redissolved in isopropanol, as the phenylalanine methyl ester was reported to be unstable in solid form.

(+)-Phenylalanine methyl ester Schiff's base of gossypol (MW 840) was obtained by mixing racemic gossypol (MW 518) dissolved in isopropanol with (+)-phenylalanine methyl

ester (MW 179) dissolved in dichloromethane. The precipitate formed was filtered out. A yellow solid was obtained in high yield and was shown to be pure by TLC analysis.

3.5.2 Chromatographic analysis.

TLC studies

Before starting the research on the separation and hydrolysis of diastereoisomers of the gossypol Schiff's bases derivatives, it was important to have already developed analytical techniques for assessing the presence of the different derivatives of gossypol, as well as to check their chemical and optical purity.

The preliminary assessment of the chemical purity of the gossypol derivative was performed by TLC on a polyamide plate, with 4:1:0.7 hexane/chloroform/HOAc as eluent. The spots were visually detected due to their bright yellow colour and non visible impurities were assessed with UV light and also with iodine vapour. Gossypol had an Rf of 0.56, the phenylalanine methyl ester Schiff's base derivative of gossypol, being less polar, eluted at a Rf of 0.67. TLC analysis could therefore be used to indicate the degree of completion of the reaction.

The optical purity of the (+)-phenylalanine methyl ester Schiff's base derivative of a gossypol sample could be approximately assessed by TLC on silica with 70/30 hexane/ethyl acetate eluent mixture. Two distinct yellow spots could be seen at Rf = 0.34 and 0.26, corresponding to the Schiff's base derivative of (-)- and (+)-gossypol respectively.

HPLC studies

The optical purity of gossypol samples was initially assessed following the published method by Matlin and Zhou.[36] First the sample was converted to the (+)-1-phenylethylamine Schiff's base derivative and resolved on a chiral HPLC column composed of the salt form of N-3,5-dinitrobenzoyl-D-(-)-phenylglycine on aminopropylsilica, monitored at 250mm with a 88:5:4:3 hexane/CH_2Cl_2/MeCN/i-PrOH mobile phase mixture. The diastereoisomer of the (-)-isomer eluted first and the purity was calculated as the percentage of the peak areas (Figure 3-7).

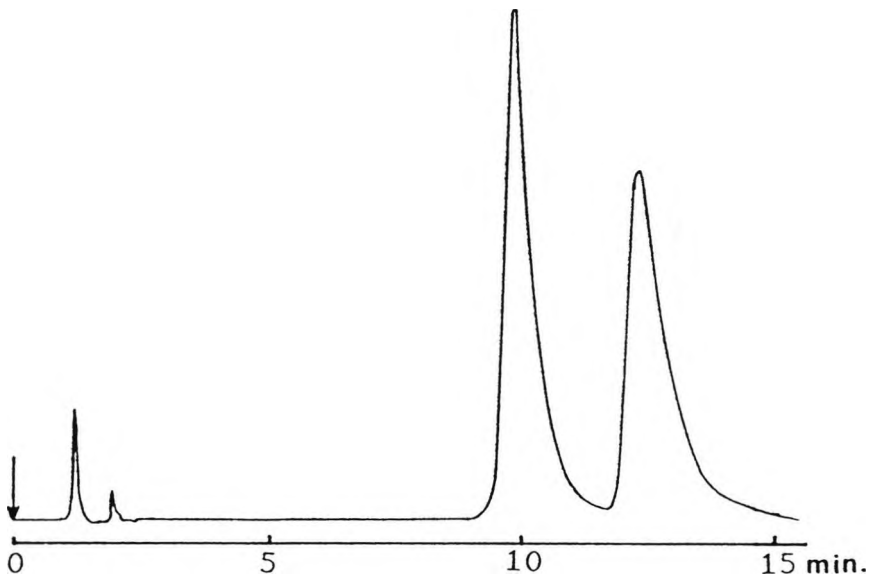


Figure 3-7
Optical purity analysis of gossypol by HPLC on a chiral column as the (+)-phenylethylamine Schiff's base derivative. Column 25 cm x 0.45 cm i.d. 5 μm N-3,5-dinitrobenzoyl-D--(-)-phenylglycine salt on Hypersil-aminopropyl silica, eluted at 2 ml/min with Hexane/CH_2Cl_2/MeCN/i-PrOH 88:5:4:3, monitored at 250 nm, 20 μl injection loop.

3.5.3 Development of chromatographic conditions specific for diastereoisomeric resolution

3.5.3.1 Normal Phase Chromatography

TLC

It was noted that (+)-phenylalanine methyl ester Schiff's base of racemic gossypol on silica TLC with 70:30 hexane/ethyl acetate solvent mixture gave two distinct spots. Initially, a medium scale TLC resolution was attempted. A high performance silica (20 x 20 cm) plate was loaded with a narrow band containing about 100 mg of the diastereoisomers.. The two bands that eluted using the eluent 70:30 hexane/ethyl acetate were partially resolved. Each band was cleanly removed, taking care not to include the overlapping band. The isolated diastereoisomer was then extracted with ether and evaporated to dryness. Both diastereoisomers were quite pure as assessed by analysis on silica TLC.

A further scaling up on preparative TLC plates using a similar eluent mixture did not achieve a good resolution. Probably the efficiency of the preparative silica plates was not high enough to resolve properly both diastereoisomers at realistic preparative loadings.

Classic column chromatography

Attention was then turned to low pressure column chromatography with the intention of increasing it later to medium pressure, if some degree of resolution had been achieved. This attempt was based on the method used by Palmer Research Laboratories to resolve racemic gossypol diastereoisomers on a gram scale. A 1m column was packed with silica. 100mg of phenylalanine methyl ester Schiff's base of racemic gossypol was applied on the top of the column eluting

it with mixture of 50:50 (v:v) hexane/ethyl acetate. A yellow band travelled down the column. Fractions were collected and their optical purities analysed by silica TLC. The first fractions showed only one spot at Rf = 0.34 belonging to the (-)-enantiomer. The proportion of hexane/ethyl acetate was gradually decreased to 15/85, the sample eluting now being only somewhat enriched in the (-)-enantiomer. With 100% ethyl acetate, enriched (+)-enantiomer was collected.

This method was not considered to be satisfactory, since at no time could two distinct bands be seen on the column, but only a continuous yellow colour. The monitoring by TLC was slow and most of the eluent fractions were only enriched. The separation itself was very time consuming. This column chromatography method was therefore abandoned.

Normal phase HPLC

Efforts were then turned to a search for an analytical method which would resolve the (+)-phenylalanine methyl ester Schiff's base of both gossypol enantiomers with a baseline separation, without using a chiral stationary phase.

Initially it was hoped that 5μ silica HPLC column would possess the required efficiency to resolve totally both diastereoisomers of gossypol, since some separation had been obtained on TLC with silica.

The initial step consisted of transferring the TLC conditions used for the resolution, i.e. 70:30 hexane/ethyl acetate (EtOAc), to an HPLC system: both diastereoisomers eluted late and unresolved. The addition of less than 5% of a solvent modifier such as acetonitrile, isopropanol (IPA) or tetrahydrofuran (THF) eluted the compound much earlier. Only the addition of THF appeared to contribute to a partial

resolution of the diastereoisomers, the best resolution being achieved with 78/17/5 hexane/ethyl-acetate/THF, the (-)-enantiomer eluting first (Figure 3-8).

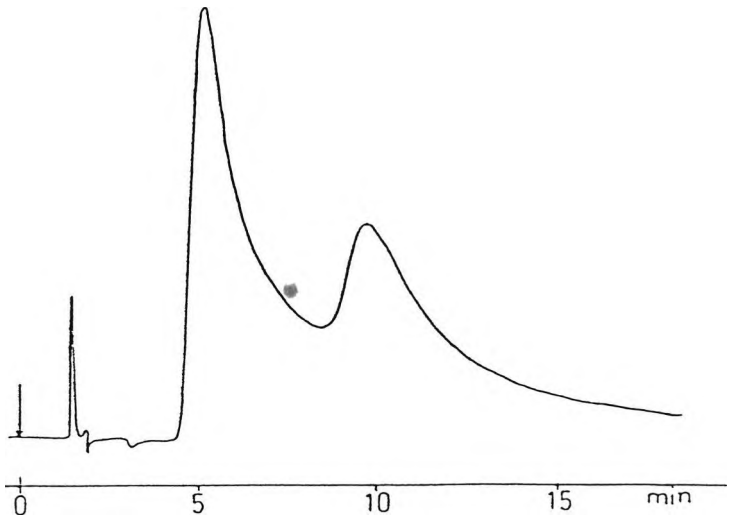


Figure 3-8
Resolution of the (+)-phenylalanine methyl ester Schiff's base diastereoisomers of gossypol.
Column 25 cm x 0.45 cm i.d. Hypersil-silica 5 µm, eluted at 2 ml/min with hexane/ethyl acetate/THF 78:17:5, monitored at 250 nm, 20 µl injection loop.

Dichloromethane was not used in the mobile phase as gossypol Schiff's bases were known to racemize in chlorinated solvents (Matlin and Zhou unpublished).

3.5.3.2 Reverse Phase HPLC

Since baseline separation of the gossypol Schiff's base derivatives of (+)-phenylalanine methyl ester could not be readily achieved on silica HPLC columns, attention was turned to the possibility of resolution under reverse phase conditions.

Resolution of the diastereoisomers was investigated on a 5µ ODS-Hypersil HPLC column. Suprisingly, when the column was injected with samples of gossypol Schiff's base of (+)-phenylalanine methyl ester and eluted with mixtures of MeOH and water, no peaks were detected for several

injections, but then the gossypol derivatives eluted only partially resolved.

The use of phosphate buffer as the aqueous system did achieve much better resolution, though not complete. The gossypol derivatives eluted as broad peaks and also tailed (Figure 3-9a). The change from methanol to acetonitrile in the mobile phase, sharpened both peaks, tailing was avoided and very good baseline resolution was obtained. A change of the pH of the phosphate buffer between 3 and 2 did not alter resolution, therefore a pH of 3 was chosen to avoid unnecessary harm to the column.

A very good and reproducible separation of the isomers was obtained with an 82:18 v/v mixture of MeCN and aqueous buffer (Figure 3-9b), the α value being 1.52 and the (-)-gossypol Schiff's base again eluting first. This is the highest degree of resolution of gossypol enantiomer derivatives so far observed on HPLC and provides an excellent system for the determination of optical purity.

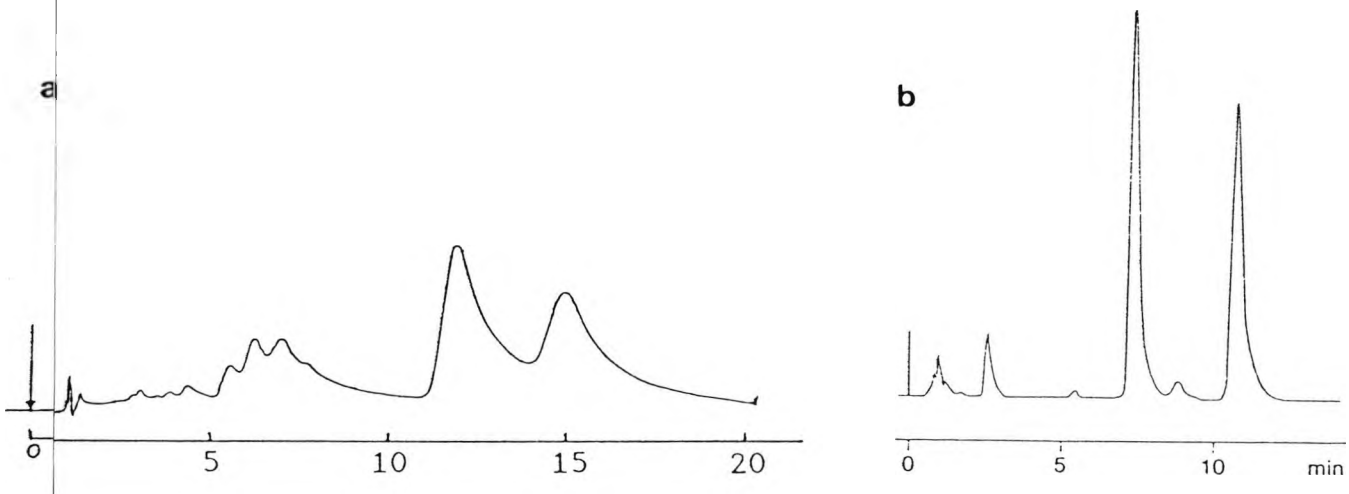


Figure 3-9
HPLC analysis of a mixture of gossypol enantiomers as the (+)-phenylalanine methyl ester Schiff's base derivatives. Column 25 cm x 0.45 cm i.d. Hypersil-ODS 5 µm, eluted at 2 ml/min with MeOH/Aq. 0.01M KH_2PO_4 (pH 3) 80:20 v/v (a), 82:18 v/v MeCN/Aq. 0.01M KH_2PO_4 (pH 3) (b), monitored at 254 nm, 20 µl injection loop.

Under the same chromatographic conditions the diastereomers of the (+)-phenylethylamine Schiff's base of racemic gossypol eluted much later and were not completely resolved (Figure 3-10).

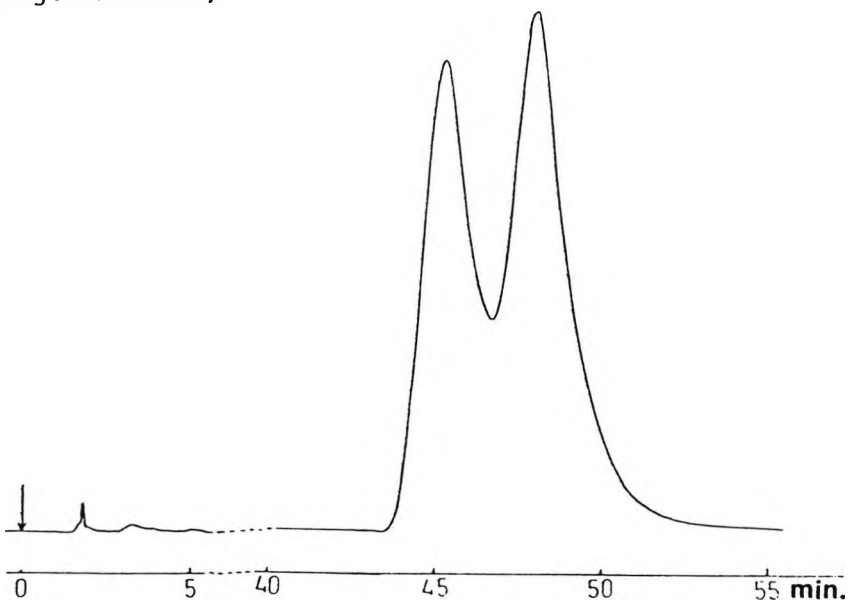


Figure 3-10
HPLC analysis of a mixture of gossypol enantiomers as the (+)-phenylethylamine Schiff's base derivatives.
Column 25 cm x 0.45 cm i.d. Hypersil-ODS 5 µm, eluted at 2 ml/min with MeCN/Aq. 0.01M KH_2PO_4 (pH 3) 75:25 v/v, monitored at 254 nm, 20 µl injection sample.

An advantage of the new method, compared with the earlier procedure using chiral columns, for the determination of optical purity is that the same column can be used, with a simple change of the proportion of constituents in the mobile phase, for the determination of chemical purity of gossypol itself (Figure 3-11).

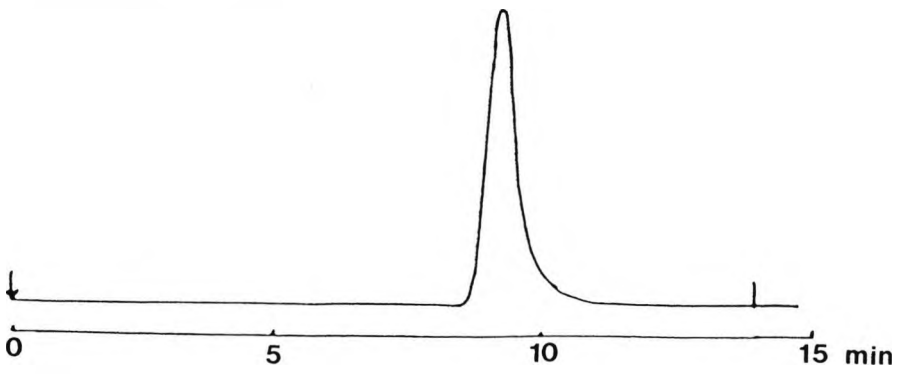


Figure 3-11
Chemical purity analysis of gossypol by HPLC.
Column 25 cm x 0.45 cm i.d. Hypersil-ODS 5 µm, eluted at 2 ml/min with MeCN/Aq. 0.01M KH_2PO_4 (pH 3) 75:25 v/v, monitored at 254 nm, 20 µl injection sample.

Part of the great advantage of this method is the easy, reliable and quick procedure of preparing this Schiff's base of gossypol compared to the time consuming preparation of the (+)-phenylethylamine Schiff's base method previously used. The reaction is carried out by mixing (1 minute sonication) a drop of the reagent solution ((+)-phenylalanine methyl ester in i-PrOH) and less than 1 mg of the gossypol sample dissolved in 1 or 2 drops of i-PrOH and final evaporation to dryness in a vacuum rotary evaporator. Before injecting in the HPLC for analysis the residue has to be redissolved in a small amount of HPLC grade acetonitrile. The reagent solution keeps stable for at least one month in the freezer, which adds to the advantages of this method.

Another advantage of this method is the possibility of monitoring the course of a Schiff's base hydrolysis reaction, as gossypol and the (+)-phenylalanine Schiff's base diastereoisomers of gossypol elute at different retention times and produce sharp and well resolved peaks.

The initial non-appearance of peaks on the first few injections of the gossypol derivatives on ODS-Hypersil is suspected to be due to the preliminary adsorption of the compounds onto the surface of the stationary phase: if the column is used on a daily basis for these compounds the isomer resolution is maintained, but if the column is then used for other work it requires reconditioning to re-establish the gossypol isomer resolution.

The possibility that adsorbed molecules may be contributing to the subsequent separation of the eluted isomers was considered. However, when the fully endcapped stationary phase Partisil-ODS3 was used instead of

Hypersil-ODS, there was no preliminary conditioning required and the isomeric gossypol derivatives eluted separately on the first injection. By contrast, the highly surface coated but non endcapped Partisil-ODS2 again gave anomalous results, with no appearance of compound peaks following the first three injections of the gossypol derivatives.

A C8-Spherisorb column was also tried. A good baseline resolution was also obtained though the peaks were much broader (Figure 3-12).

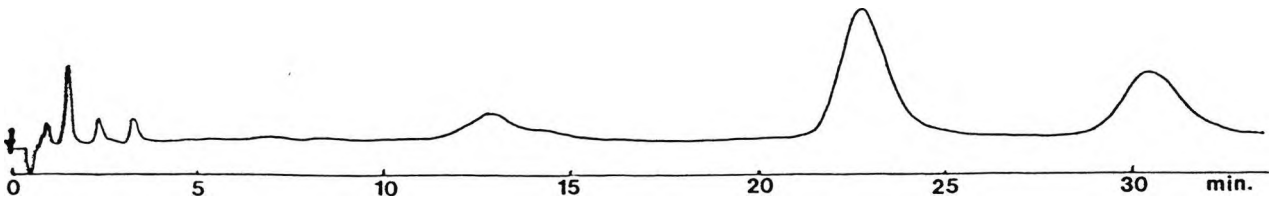


Figure 3-12
Resolution of gossypol enantiomers as the phenylalanine methyl ester Schiff's base derivative.
Column 25 cm x 0.45 cm i.d. Spherisorb C-8 10 µm, eluted at 2 ml/min with MeCN/Aq. 0.01M KH_2PO_4 (pH 3) 65:35 v/v, monitored at 254 nm, 20 µl injection sample.

3.5.3.3: Semipreparative and Preparative Reverse Phase HPLC Separation

The large α value obtained in the isomer separation on ODS-Hypersil indicated that this stationary phase would be well suited to scale-up for preparative HPLC. At an intermediate scale, a semipreparative column (30 cm x 7 mm i.d.) gave complete resolution of the isomers at a 16 mg loading, with a small impurity peak appearing between the two main components (Figure 3-13).

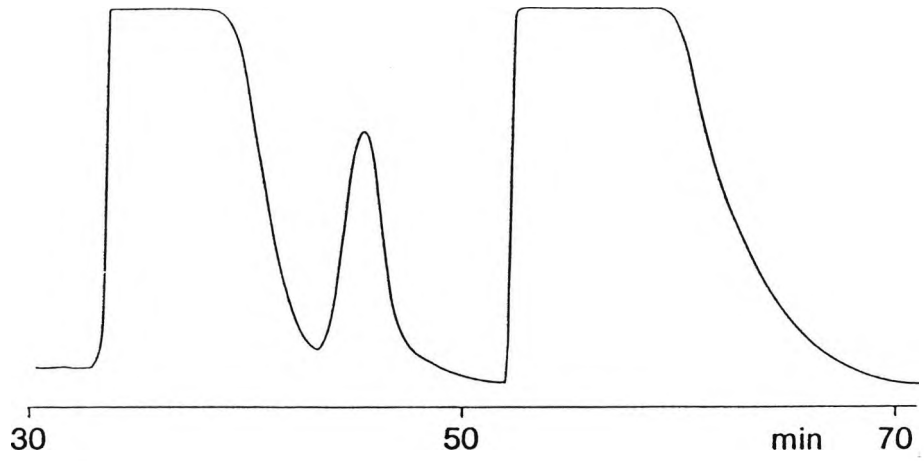


Figure 3-13
Semipreparative HPLC separation of the (+)-phenylalanine methyl ester Schiff's base diastereoisomers of gossypol. Column 30 cm x 0.7 cm i.d. Hypersil-ODS 5 µm, eluted at 4.5 ml/min with MeCN/Aq. 0.01M KH_2PO_4 (pH 2) 72:28 v/v, monitored at 254 nm x 2 AUFS. Injection of 16 mg in 40 µl CH_2Cl_2.

A large preparative column (33 cm x 22 mm i.d.) was then constructed, for economy the packing material being synthesized in our laboratory by reaction of 5 µm Hypersil with octadecyltrichlorosilane.[40] On this column, after preliminary conditioning as discussed above, 100 mg of the gossypol Schiff's base isomer mixture cold be separated in one run taking just over 1 hour (Figure 3-14).

The separated compounds were recovered from eluted HPLC fractions by vacuum concentration at 40°C and extraction with ether.

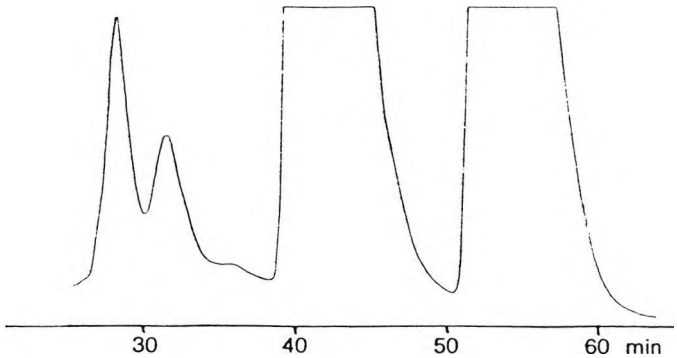


Figure 3-14
Preparative HPLC separation of the (+)-phenylalanine methyl ester Schiff's base diastereoisomers of gossypol. Column 33 cm x 2.2 cm i.d. Hypersil-ODS 5 µm, eluted at 8.5 ml/min with MeCN/Aq. 0.01M KH_2PO_4 (pH 2) 82:18 v/v, monitored at 254 nm x 2 AUFS. Injection of 100 mg in 2 ml MeCN.

3.5.4 Hydrolysis Studies

Determination of the optical purity of samples recovered from both the silica column and reverse phase preparative HPLC separations of the Schiff's base isomers was carried out by injection onto an analytical Hypersil-ODS column.

An accumulated batch of more than 70 g of the (-)-gossypol Schiff's base manufactured by Palmer research laboratories by classical silica column chromatography was found to be between 80 and 85% chemically pure and 95-99% optically pure (Figure 3-15a). Similarly a batch of 20 g of the (+)-gossypol Schiff's base was determined as about 92% chemically pure and 98% optically pure (Figure 3-15b).

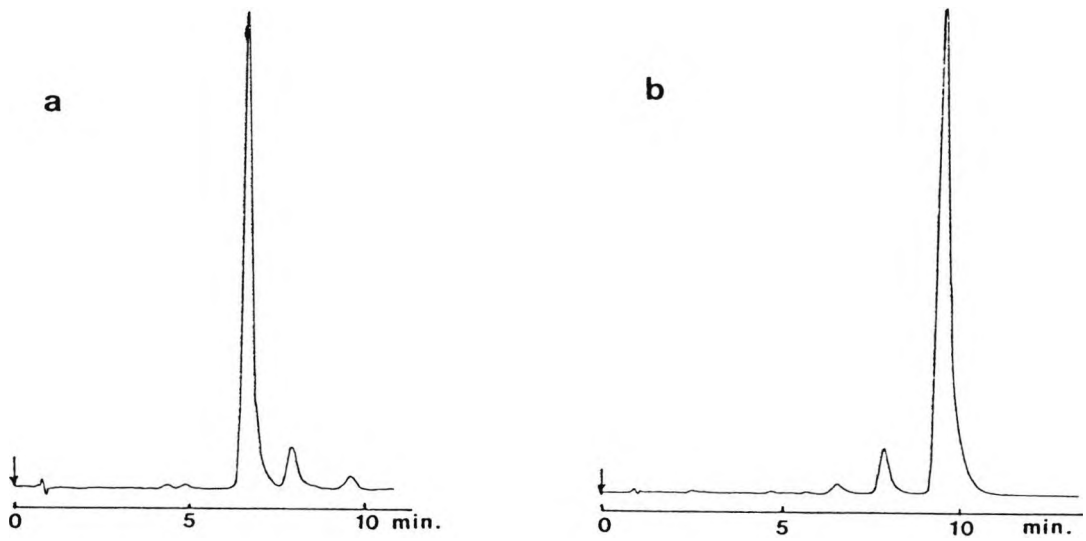


Figure 3-15
Simultaneous chemical and optical purity analysis of the (+)-phenylalanine methyl ester Schiff's base derivatives of the enriched a) (-)-enantiomers, and b) (+)-enantiomer of gossypol supplied by Palmer research laboratories.
Column 25 cm x 0.45 cm i.d. Hypersil-ODS 5 µm, eluted at 2 ml/min with MeCN/Aq. 0.01M KH_2PO_4 (pH 3) 82:18 v/v, monitored at 254 nm, 20 µl injection sample.

Alkaline Hydrolysis

Initially an alkaline hydrolysis was attempted following the hydrolysis procedure previously used for the (+)-phenylethylamine Schiff's base derivative of gossypol published by Matlin and Zhou.[36]

Treatment of the (+)-phenylalanine methyl ester Schiff's base derivative of gossypol with aqueous KOH and acetonitrile at 30°C yielded a dark product. A diluted solution of dithionite ($Na_2S_2O_4$) had been added during the hydrolysis reaction to avoid the oxidation of the hydroxy groups. After working up the hydrolysis products, the analysis on a polyamide TLC plate gave a complex mixture. Apparently only partial hydrolysis of the imine and ester function had occurred. Neither increase in the temperature of the reaction (up to 70°) nor in the duration of the hydrolysis improved the hydrolysis yield.

Acid Hydrolysis

Due to the failed attempts to regenerate gossypol cleanly by alkaline hydrolysis, acid hydrolysis conditions were examined. Following the published hydrolysis procedure,[42] gossypol was completely regenerated using a cold mixture of acetic acid, sulphuric acid, water and ether. The (+)-phenylalanine Schiff's base derivative of gossypol was initially dissolved in equal amounts of ether and glacial acetic acid. After cooling to below 0°C and acidifying with concentrated sulphuric acid, the hydrolysis was initiated on the addition of water. This reaction proved stable if the reaction was kept cold (<3°C) continuously.

The success of the reaction was monitored by analysing on Polyamide TLC plates the worked up product of an aliquot of the hydrolysis mixture. After several hours, the starting material had been cleanly and completely converted to gossypol, and yellow crystals could be collected. Further crops of gossypol could be separated from the mother liquor after it had been kept several hours in the refrigerator.

The degree of epimerization was studied at different stages of the hydrolysis procedure and monitored by analytical reverse phase HPLC. Neither the dissolution of the gossypol derivative in ether and glacial acetic acid nor the prolonged presence of concentrated sulphuric acid at low temperature caused any significant epimerization. No gossypol was formed in the absence of water.

The maintenance of low temperature (<3°C) throughout the hydrolysis proved to be a critical factor. An increase to ambient temperature resulted in the formation of a black crust. Low temperature (down to 3°C) could be maintained by immersing the reaction vessel in an ice bath containing salt.

Various parameters were studied which affected the yield of the hydrolysis, such as starting the hydrolysis reaction by the addition of water, the right amount of concentrated sulphuric acid and water and the required proportion of solvent used to dissolve the gossypol derivative.

Water was added 30 minutes and 24 hours after concentrated sulphuric acid was introduced. As a 30 minute interval achieved the highest yield, after additional small scale experiments, it could be concluded that hydrolysis should be started simultaneously or just after the addition of the sulphuric acid.

Gossypol (+)-phenylalanine methyl ester Schiff's base could not be dissolved in less solvent than the ratio 1: 12: 12 w/v/v gossypol derivative/ether/glacial acetic acid. Two experiments were carried out with a ratio of reagent 4: 4: 1 and 4: 4: 4 of ether/glacial acetic acid/conc. sulphuric acid. A higher yield of regenerated gossypol was achieved when the former ratio was applied. Unnecessary addition of concentrated sulphuric acid was only counterproductive.

Following a careful work up procedure of various aliquots at different hydrolysis reaction times as described in the Experimental section 3.6, the HPLC chromatograms provide quantitative results for monitoring of the hydrolysis (Scheme 3-6).

When the course of hydrolysis was followed by HPLC, it was observed that a new peak (Peak B) appeared at a retention time intermediate between those of the Schiff's base (Peak A) and free gossypol (Peak C) (Scheme 3-6). This peak, which increased in size and then declined as hydrolysis proceeded (Figure 3-16), is evidently the intermediate corresponding to cleavage of one of the two imine functions (Scheme 3-7).

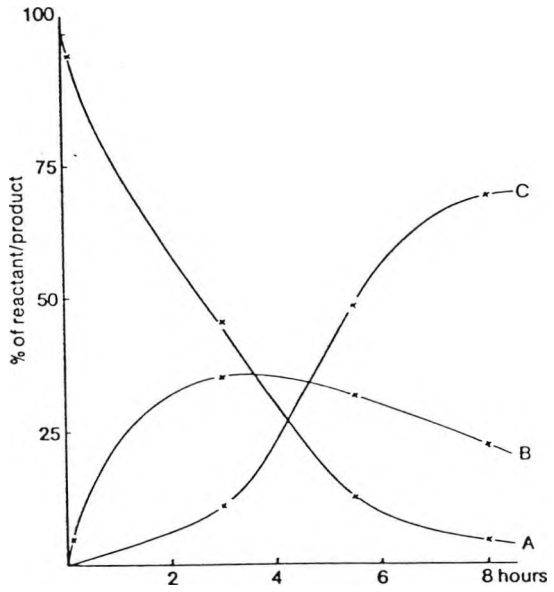
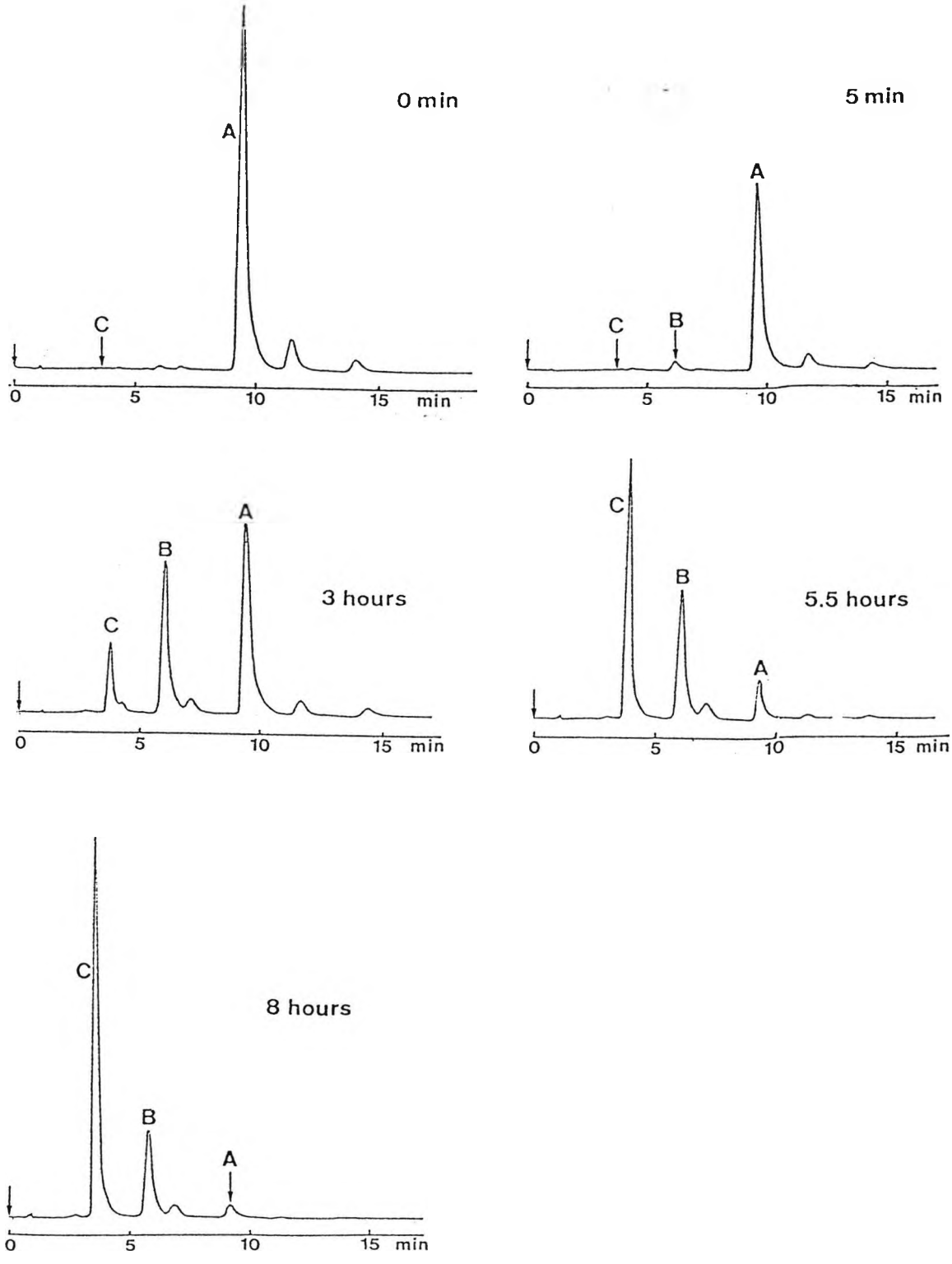
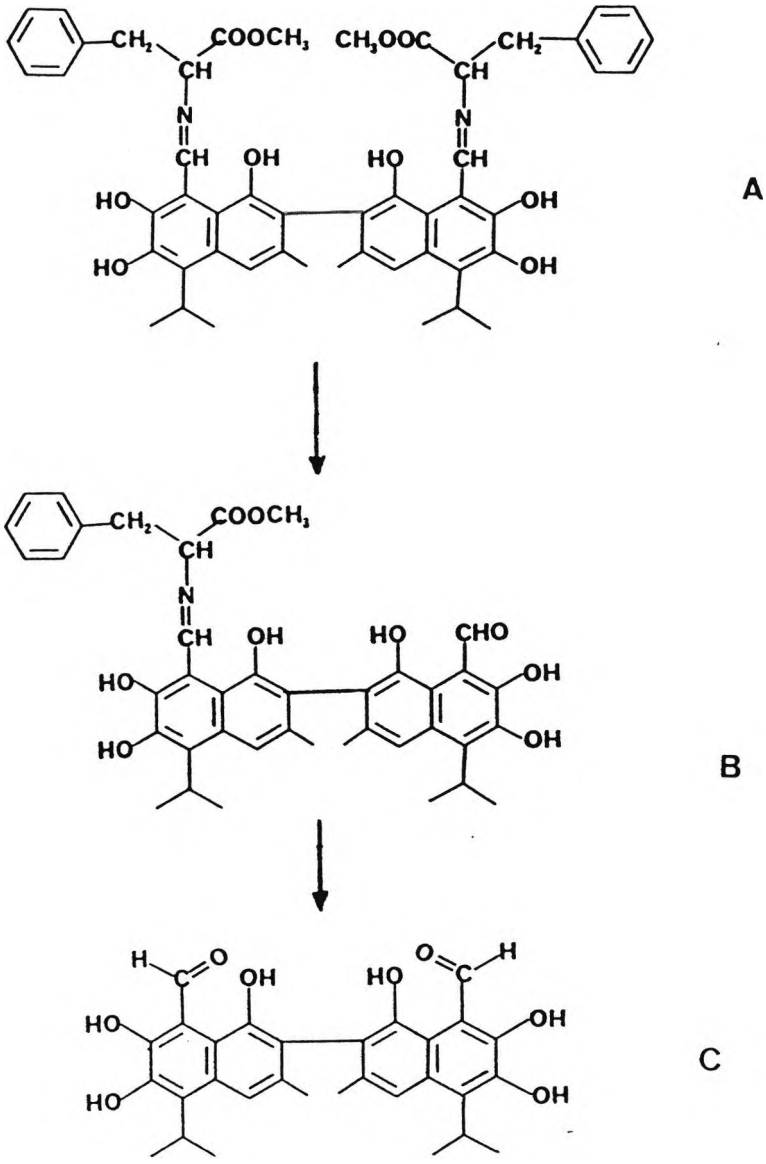


Figure 3-16
Time course of hydrolysis of (+)-phenylalanine methyl ester Schiff's base diastereoisomers of (-)-gossypol (A); (B) intermediate; (C) gossypol.



Scheme 3-6
HPLC of reaction mixture during acid hydrolysis of (+)-phenylalanine methyl ester Schiff's base diastereoisomers of (-)-gossypol (Peak A); (Peak B) intermediate; (Peak C) gossypol at different periods .
Column 25 cm x 0.45 cm Hypersil-ODS 5 um, eluted at 2 ml/min with MeCN/Aq. 0.01M KH_2PO_4 (pH 3) 82:18 v/v, monitored at 254 nm, 20 ul injection sample.



Scheme 3-7
Stepwise hydrolysis of gossypol Schiff's base via a mono-imine intermediate: (A) diastereoisomer of (-)-gossypol, (B) mono-imine intermediate and (C) gossypol.

3.5.5 Fractional Crystallization

On hydrolysis of the (-)-gossypol-enriched Schiff's base, it was observed that the liberated gossypol crystallized slowly from the mixture over a period of hours after the end of the reaction. When fractions of crystals were collected at intervals of time, the earliest precipitate had a uniform composition of ca. 45:55 (+)/(-)gossypol until nearly all the (+)-isomer had been removed, and the subsequent fractions were found to be increasingly pure (-)-gossypol. The inverse result was obtained on hydrolysis of the (+)-gossypol-enriched Schiff's base.

An addition of small amounts of water to the mother liquor when pure crops where being collected increased the yield of gossypol. Care had to be taken not to add to much water as an oil would be formed. The gossypol crops were collected by filtering the solution through a filter funnel under vacuum. During this filtration, some ether evaporated from the mother liquor, therefore concentrating the solution and contributing to the formation of the next crop. Immediately after filtering, the mother liquor was replaced in the refrigerator each time; the residue was washed with freshly prepared dilute aqueous sodium dithionite solution to avoid oxidation of the hydroxyl groups of gossypol. The gossypol crop was subsequently dried under vacuum.

After all the crops had been collected, the residual mother liquor was evaporated down and then triturated with hexane. The yellow residue obtained was first dissolved in dry acetone and evaporated down to dryness to increase solubility. Recrystallization was performed in ether/glacial acetic acid. The crops obtained were unfortunately chemically very impure though optically totally pure.

Gossypol samples with 99.5% to 100% content of a single enantiomer were obtained by carefully following this fractional crystallization procedure.

This was achieved by taking advantage of the fact that racemic gossypol can be crystallized from solution as its relatively insoluble 1:1 acetic acid complex. Thus, when an 80:20 mixture of gossypol enantiomers was dissolved in ether/acetic acid, the racemic acid complex crystallized out and the mother liquors were concentrated to small volume and triturated with hexane, giving a precipitate of the excess enantiomer in a chemically pure state.

Using this combination of techniques, the recovery of optically pure samples of each gossypol enantiomer could be maximized and the racemic material recycled.

3.5.6 Micronization

All the batches of optically and chemically pure (-)-gossypol, collected from the 3 centre study, were checked on the particle size analyser for particle size distribution.

The initial material of pure (-)-gossypol had 50% of the particles higher than 50 µm and 35% higher than 100 µm, thus forming a very wide non-gaussian distribution.

Milling was the first technique attempted for micronization. After 14 hours of dry milling, the larger particles had decreased in size though still giving a very wide non-gaussian distribution. Wet milling with hexane did not improve the micronization.

An alternative method for micronization is precipitation. The dropwise addition of 40 g of a nearly saturated ethanolic solution of (-)-gossypol to vigorously stirred cold water precipitated gossypol which, after drying under vacuum, complied with the desired particle size characteristics, suitable for the intended toxicology studies (Figure 3-20).

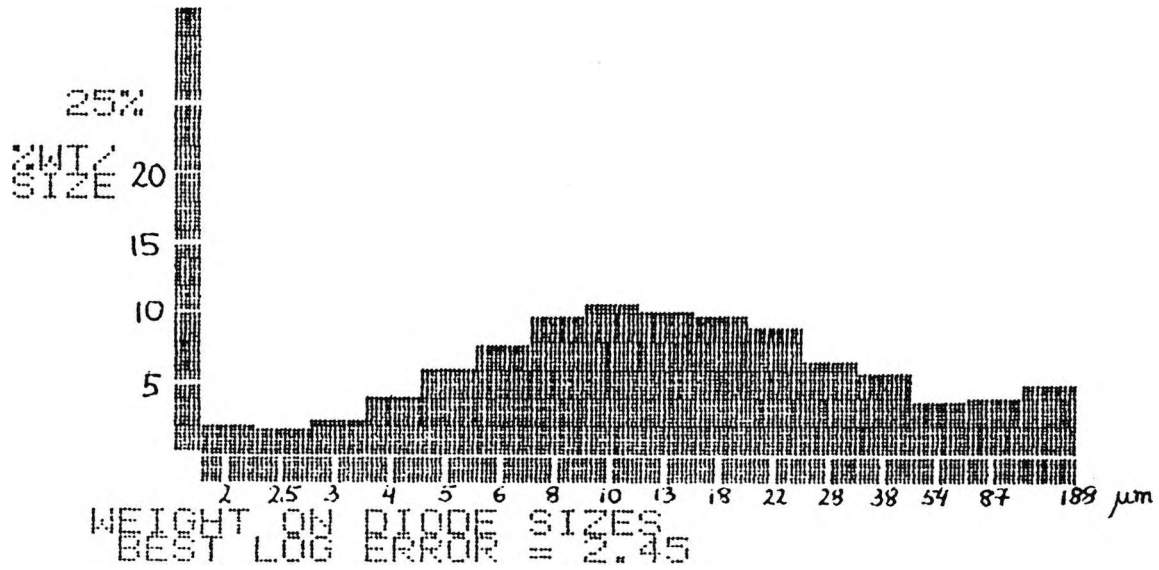


Figure 3-20

Particle size distribution of pure (-)-gossypol

3.6 Experimental

HPLC

Analytical separations were run using a Waters 6000a pump, Rheodyne 7125 injector (20 µl loop), and Cecil 2112 variable wavelengh detector (8 µl flowcell) at 250 nm connected to a Linseis recorder and to a Trivector TRIO integrator. Analytical columns were constructed of 25 cm x 0.45 cm i.d. and semipreparative columns of 30 cm x 0.7 cm i.d. 316 stainless steel, high pressure slurry packed with the stationary phase.

For preparative HPLC, a Q1 Metripump (Metering Pumps Ltd, London) was used, the Rheodyne injector fitted with a 4 ml loop and the detector with a preparative (1 mm pathlengh) flowcell.

The preparative HPLC column packing was synthesized by refluxing octadecyltrichlorosilane (100 g) with 5µm Hypersil (290g) in dry toluene (1.2 l) and dry pyridine (300 ml) overnight. The solid was filtered, washed with dry toluene, acetone and methanol, followed by methanol/water 1:1 and acetone. After vacuum drying at 40°C, the product (C 10.4%, corresponding to 2.1 groups/nm²) was high pressure slurry packed in methanol:isopropanol 8:2 v/v into a 33 cm x 2.2 cm i.d. preparative column.

Chiral bonded phase

Aminopropyl silica, prepared by reaction of Hypersil 5µm silica with 3-aminopropyl triethoxisilane, was stirred with a solution (0.5 Mol) of the chiral acid N-(3,5-dinitrobenzoyl-D-(-)-phenylglycine (1.7 mMol per 1 g aminopropyl silica) in THF.

Synthesis of Gossypol Schiff's Base

Racemic gossypol (0.0432 Mol) was stirred at room temperature for 2 h with (+)-phenylalanine methyl ester (0.162 Mol) in isopropanol (250 ml) and the product filtered off, washed with cold methanol and vacuum dried to give the Schiff's base (30.3 g, 83.4%) as a yellow solid, m.p. 139-145°C. A second crop (4.8 g, 13.2%) was obtained on cooling the filtrate to 0°C overnight. Both crops exhibited two spots (R_f 0.34 and 0.26) on TLC (Merck precoated Silica 60 F_{254} plates, hexane/ethyl acetate 70:30 v/v). After separation, either by silica column chromatography or by preparative reverse phase HPLC as discussed in the text, the first eluted diastereoisomer was a yellow solid, m.p. 85-88°C, found: C 70.65%, H 6.39%, N 4.07% ($C_{50}H_{52}N_2O_{10}$ requires C 71.41%, H 6.23%, N 3.33%).

Hydrolysis

To a solution of the resolved gossypol(+)-phenylalanine methyl ester Schiff's base (10.0 g, 11.9 mMol) in distilled ether (120 ml) and glacial acetic acid (120 ml) stirred and cooled to -1°C were added concentrated sulphuric acid (20 ml) and water (40 ml). After 7 h, the first crop of solid gossypol was filtered off with vacuum suction and washed with 0.01% w/v aqueous $Na_2S_2O_4$ and the filtrate returned to the refrigerator and repeatedly refiltered at intervals of 2 h to produce further crops of gossypol.

Work up Procedure for monitoring Hydrolysis Procedure

To an aliquot (0.2 ml) of hydrolysing solution transferred to a flask (10 ml), aqueous saturated $NaHCO_3$ was added (2-3 ml) to neutralize until effervescence ceased. After addition of ether (2.0 ml), the extraction was carried out by agitating carefully with a Pasteur pipette. After the aqueous phase was removed the ether solution was evaporated with an air flow and redissolved in HPLC grade acetonitrile (1.0 ml) for injection onto the ODS-Hypersil column.

HPLC Analysis of Optical Purity of Gossypol

To a sample of resolved gossypol (1 mg) in 3 drops of isopropanol was added 1 drop of a solution of (+)-phenylalanine methyl ester in isopropanol (0.25 g/ml; stored at -10°C). After sonication to aid mixing, the solution was kept for 1 minute, evaporated to dryness under vacuum, and redissolved in acetonitrile for injection onto the ODS-Hypersil column.

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CHAPTER 4

TRIPTERYGIUM WILFORDII

4.1 Origin of the project

Tablets containing an extract of Tripterygium wilfordii Hook f. (Celastraceae) are used in traditional Chinese medicine for the treatment of a variety of ailments, including rheumatism, ankylosing spondylitis and dermatological conditions.

During a WHO site visit to Nanjing in 1985 and subsequently at a WHO Task Force Steering Committe meeting early in 1986, Dr. Qian Shao Zhen revealed that there were preliminary indications that the TW tablets could cause infertility in male animals. This offered the potential of providing a new lead for the development of an agent for male fertility regulation and the WHO Task Force decided in April 1986 to support work on the isolation and identification of the active constituents present in the plant extract.

Zhang Jian Wei, a chemist from Dr. Qian's institute in Nanjing, started work at the beginning of 1986 in the City University under the supervision of Professor Matlin on the phytochemistry of Tripterygium wilfordii on a WHO grant. Moreover, the Steering Committee also decided to support a research fellow at City University from mid-1986. Vivien Stacey carried out the initial HPLC separation of TW from mid. 1986 to mid-1987, when I joined this project until mid-1989. I did further subfractionation of the TW material isolation and identification of all the active constituents responsible for the antifertility action of the plant extract. Fractions and compounds isolated were sent from London to Nanjing, where Dr. Qian's colleague, Xu Ye, carried out in vivo testing in male rats.

4.2 Plant material

It was recognized at the outset that an important factor affecting the success of the project would be the availability of an adequate supply of material for investigation. At the beginning of this work, an agreement was reached between WHO and the Chinese authorities, the latter undertaking to supply at least 500 g of crude extract of Tripterygium wilfordii for the work at the City University. However, this material was not provided and Professor Matlin's group was only able to proceed with the project by obtaining, some batches of TW tablets over a period of 1-2 years and, at a later stage, some batches of "extracts" of the plant, of ill-defined mode of preparation. It must be emphasized that the gradual acquisition of successive, small batches of materials by Professor Matlin's group posed a strategic problem: it was necessary to proceed in a cautious, linear fashion on the subfractionations, in order not to waste precious material before bioassay results were obtained; it was also necessary to reconfirm activity and potency with each fresh batch that appeared, causing further delays; there was a degree of inconsistency in content between various materials - specially between the tablets and the later Chinese "extracts", which also has led to problems in gaining access to some of the components of interest.

Very little information is available about the preparation of the tablets, but it is understood that they contain 10 mg/tablet of material which is obtained from an aqueous-soluble fraction: 10 mg of residue of the aqueous phase is claimed by one source to be equivalent to 25 g of crude root xylem before extraction. Other sources suggest

that the extract is made from bark rather than xylem. This is combined in the tablets with excipients which may include starch and magnesium stearate.

4.3 Tripterygium wilfordii, its chemical constituents and biological properties: literature review.

Tripterygium wilfordii Hook f., called 'lei kung teng' or 'thunder god vine', by the Chinese, is a perennial twinning vine belonging to the family Celastracea with foliage and manner of growth very much like that of the North American bittersweet, Celastrus scandens L. The fruit of Tripterygium is dry, yellowish green or brownish in colour, 1.5-2 cm long and 7-10 cm wide, with 3 wings (Fig. 4-1).

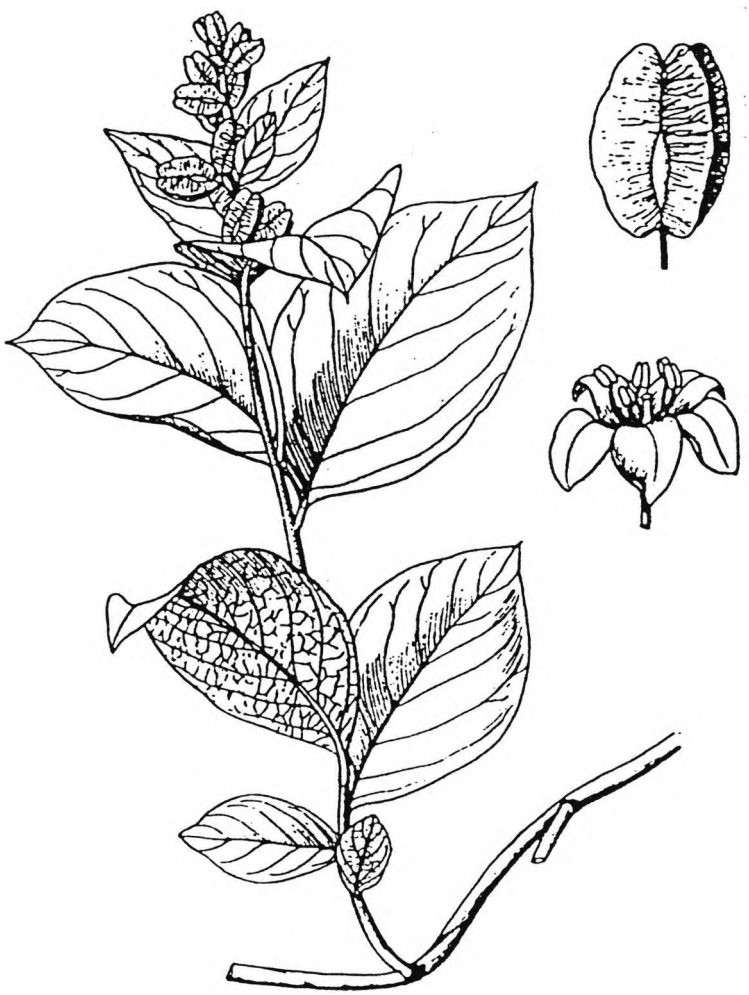


Fig. 4-1: Tripterygium wilfordii Hook

4.3.1 Alkaloids

Sesquiterpene alkaloids

Traditionally, Tripterygium wilfordii was cultivated rather widely in several Chinese provinces, Chinese market gardeners making use of the powdered roots as an insecticide to kill chewing insects which eat the leaves of vegetables such as cabbage, cucumber, carrots, etc.

The insecticidal properties of Tripterygium wilfordii began to attract a marked interest amongst Chinese scientists in 1931 and several papers were published by Chinese workers on this topic. Some of these reports provided evidence of its insecticidal value and properties as a stomach poison.[1-6]

The translation of some of these Chinese papers attracted interest amongst Western scientists and reports began to appear in the Western literature during the 1940's.[7-11] The U.S. Plant Introduction Garden of the Division of Plant Exploration and Introduction at Glenn Dale, Maryland, showed great interest in the potential of this plant. Several thousand cuttings of Tripterygium wilfordii were sent in 1935 from China to the U.S. where they grew very well in the Glenn Dale Laboratories. The first tentative test carried out by Haller, Siegler and Swingle from the Division of Control Investigations, Bureau of Entomology and Plant Quarantine with root powder sent from China in 1935 proved to have little or no value as a contact insecticide.[7] In 1936, a second root powder batch from China proved equally unsuccessful as a contact insecticide. The powders were up to six months old when tested, and the Chinese insisted that the insecticidal effect of this powder declines steadily and is entirely lost after one year storage.

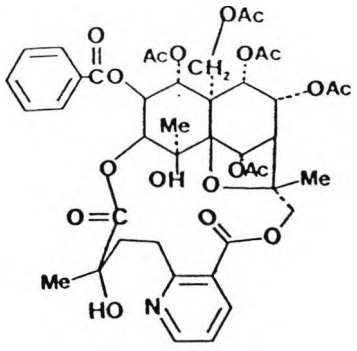
In view of the indecisive nature of these preliminary tests of more or less deteriorated powders, further tests were initiated in 1939 with freshly prepared powder from the plants growing at the Plant Introduction Garden at Glenn Dale. This material proved to be quite potent as a contact insecticide. Alcohol extracts gave slightly better results.[7]

The insecticidal principle from Tripterygium wilfordii was reputed by the Chinese pioneer scientists to be an alkaloid, but little was known regarding its nature. In 1937 Chou, Hsu and Hwang[12] isolated the toxic alkaloid fraction which they named tripterygine. In 1939 Hwang[13] assigned to tripterygine the formula $C_{32}H_{38}O_{11}N_1$.

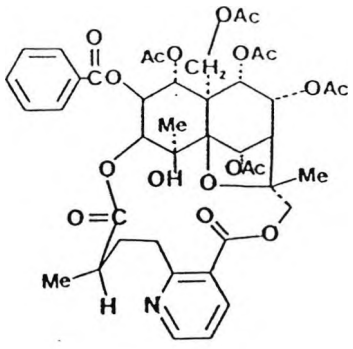
In 1950 Acree and Haller[14] reported the isolation of an alkaloid from an ether extract of the root, which they named 'wilfordine' and it was found to be an ester alkaloid which yielded eight equivalents of acid upon saponification with ethanolic potassium hydroxide.

Craig[15] reported that Acree's wilfordine had been shown by countercurrent distribution to be a mixture.

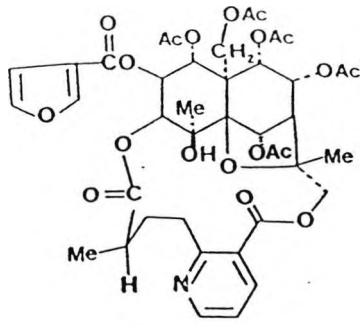
Starting in 1950, Beroza[16-20] isolated by partition chromatography the toxic components of Acree and Haller's wilfordine and named them wilfordine (1), wilforine (2), wilforgine (3) and wilfortrine (4). A weaker insecticidal alkaloid was also isolated by Beroza and was named wilforzine (5).[20,21]



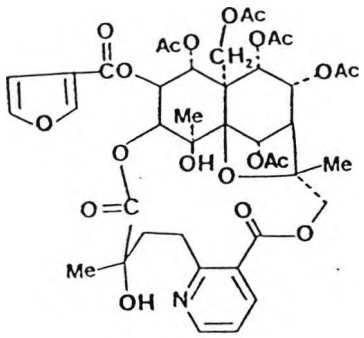
(1)
Wilfordine



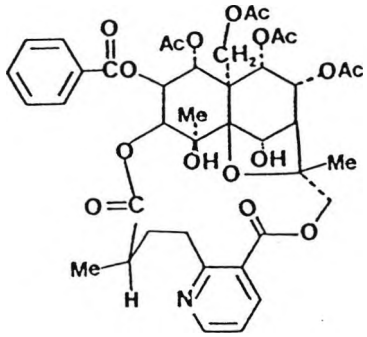
(2)
Wilforine



(3)
Wilforgine



(4)
Wilfortrine

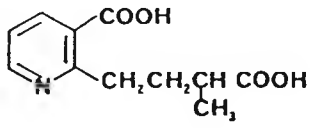


(5)
Wilforzine

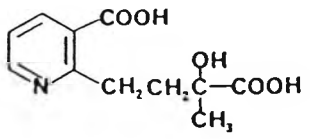
Beroza found the five alkaloids to have molecular weights of the order of 900. Structural studies showed that the five alkaloids contained the same polyhydroxy nucleus. Apart from wilforzine, the four other alkaloids contain 10 hydroxy groups, 8 of which are esterified in the intact alkaloid, 6 with steam volatile acid, the residual 2 hydroxyls are esterified to a nitrogen-containing non steam volatile dicarboxilic acid.[19,20]

Saponification of wilforine and wilfordine yielded the same 6 volatile acids: 5 mol of acetic acid and 1 mol of benzoic acid, similar to wilforzine which yielded only 4 mol of acetic acid and 1 mol of benzoic acid. Saponification of wilforgine and wilfortrine yielded 5 mol of acetic acid and 1 mol of 3-furoic acid as their 6 volatile acids.[19,20] The 3-furoic acid is a rare acid that had been isolated from a natural source only twice before.[22]

Alkaline saponification of the 5 alkaloids yielded amongst other products two pyridine dicarboxylic acids: wilfordic acid (6) found in wilforine, wilforgine and wilforzine and hydroxywilfordic acid (7) found in wilfordine and wilfortrine.[21]



(6)
Wilfordic acid



(7)
Hydroxywilfordic acid

Initial X-ray diffraction pattern studies indicated that all 5 alkaloids had the same polyhydroxy nuclei,[19] and an NMR study suggested that this C15 polyhydroxy nucleus was composed of 3 fused rings.[21]

Later Shizuri et al[23] analysed the NMR spectra of the polyhydroxy terpenoid nucleus of the Celastraceae alkaloids and Taylor and Watson[24] proved that it consists of two trans fused six membered rings and a five membered cyclic ether formed by fusion of two axial substituents. In 1987, He et al[25] also discussed the structures of these alkaloids.

The four major alkaloids - wilforine, wilfordine, wilforgine and wilfortrine - account for about 90% of the total alkaloids in Acree and Haller's "wilfordine".

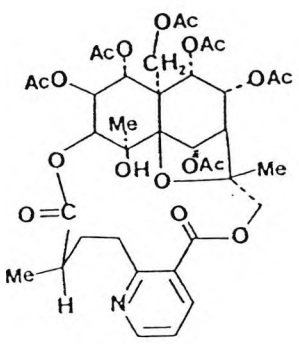
Toxicology tests of a 60 ppm solution of each of the four main alkaloids proved them to have a lethal effect on the newly hatched larvae of the European corn borer within 3 days. Wilforzine, however exhibited definite but much less insecticidal action which could indicate that the insecticidal activity of the alkaloid may depend upon the ester groups being intact.[22]

No significant biological activity has been reported for the polyhydroxy sesquiterpene nucleus itself. However, it has not been determined whether the polyhydroxy moieties might serve as inert carriers of the alkaloid fragment, as active transport agents or as orienting groups for the alkaloid fragment at the active site.[24]

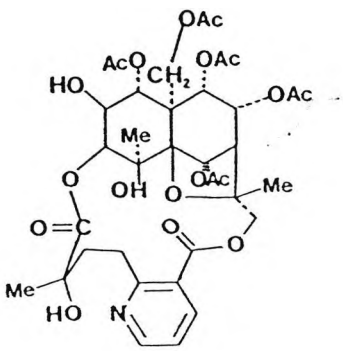
Tripterygium wilfordii was found to be non toxic to the housefly or to warm blooded animals.[22] Literature has been published about the characterization of other alkaloids of the celastraceae family such as maytoline,[24] and evonine.[24,26]

Additional analytical,[27] biosynthetic[28,29] and structural studies[21] of these alkaloids were also carried out.

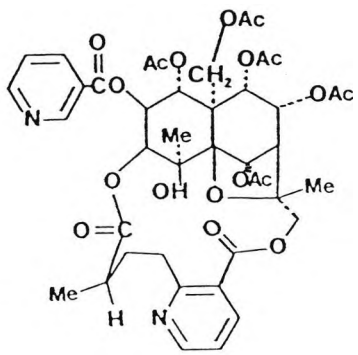
In the last few years, three new sesquiterpene alkaloids have been isolated from Tripterygium wilfordii and identified, namely wilforidine (8),[30] euonine (9) also named wilformine,[25,31] and wilfornine (10).[31] These last two compounds have been found to possess immunosuppressive activity in mice.[31]



(8)
Wilforidine

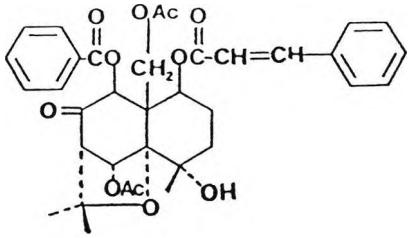


(9)
Eunonine

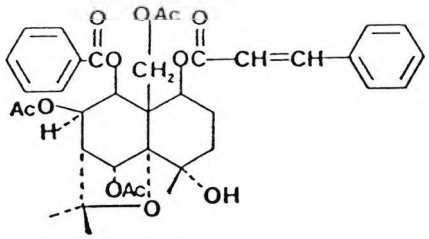


(10)
Wilfornine

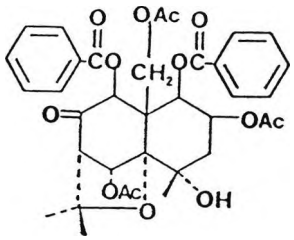
A further three new polyhydroagaro derivatives were also isolated from the leaves of Tripterygium wilfordii and named triptofordin D-1 (11), D-2 (12) and E (13), additionally to two related esters (14) and (15).[32]



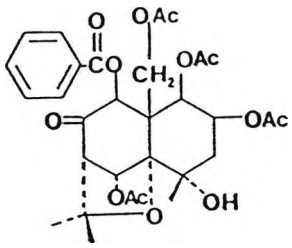
(11)
Triptofordin D1



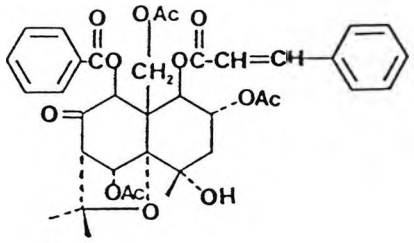
(12)
Triptofordin D2



(13)
Triptofordin E



(14)



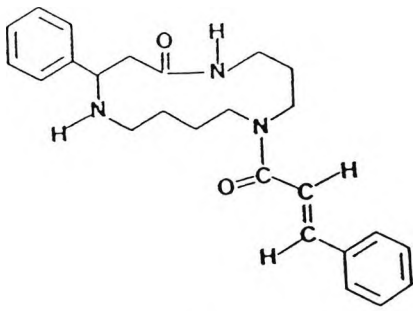
(15)

Macrocyclic spermidine alkaloids

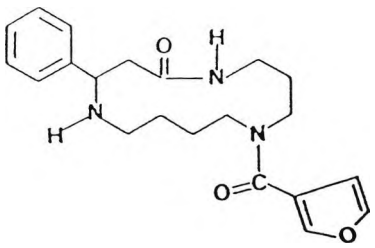
During subsequent phytochemical investigations Kupchan et al amongst other scientists isolated and identified a new group of macrocyclic spermidine alkaloids and the lignan syringaresinol.[33-35]

Celacinnine, initially isolated from Maytenus arbutifolis,[26] and later from Tripterygium wilfordii is the prototype of a novel series of alkaloids present in members of the Celastraceae family. The alkaloid (16) is characterized by the presence of a 13-membered ring containing spermidine.[36]

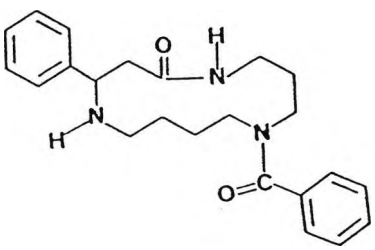
Kupchan et al[34,35] and Wagner et al[37] isolated and characterized by chemical degradation,[34] and by spectral data,[35,37] celacinnine (16) and two other related alkaloids: celafurine (17) and celabenzine (18). The three alkaloids differ only in the acyl residue on the C-5: trans-cinnamic acid, furoic acid and benzoic acid respectively.



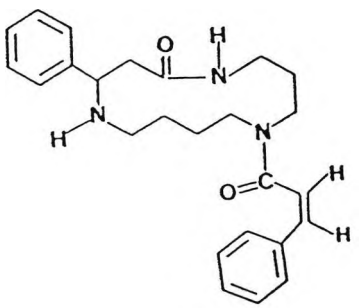
(16)
Celacinnine



(17)
Celafurine



(18)
Celabenzine



(19)
Celallocinnine

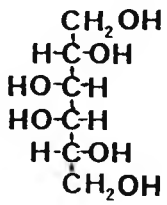
The cis-isomer of celacinnine (16), named celallocinnine (19) was isolated also from twigs and leaves of Maytenus arbutifolia. By irradiating celacinnine with a long wavelength UV light, celallocinnine is obtained in a good yield, where a trans to cis isomerization of the cinnamoyl residue takes place. It is therefore conceivable that cis-isomer formation occurs "in vivo" in the leaves under the influence of sunlight.[38]

The hopes of antitumour potential of the spermidine macrocyclic alkaloids were diminished when rats treated with an analogue alkaloid, maytosin, developed acute gastrointestinal damage amongst other problems.[39,40]

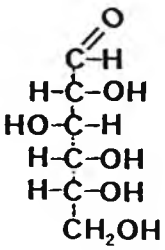
4. 3. 2 Miscellaneous components

Sugars

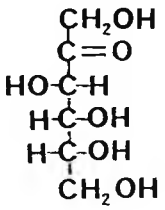
The earlier efforts by Acree and Haller to isolate the insecticidal toxic principle from the ground whole roots of the plant resulted in the isolation of the sugar 'dulcitol' from both the aqueous and the ethanolic extracts.[14] Dulcitol (20) together with glucose(21) and fructose(22) had been isolated previously by Chinese scientists in 1936.[2]



(20)
Dulcitol



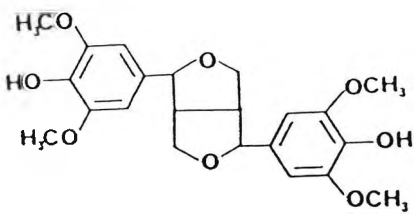
(21)
D-(+)-glucose



(22)
D-(-)-fructose

Lignans

Kupchan et al[41] isolated (-)-syringaresinol in the course of a continuing phytochemical examination. Syringaresinol (23) is a member of a substantial group of lignans having a 2,6-diaryl-cis-3,7-dioxabicyclo-(3,3,0)-octane structure. The lignans differ from one another principally in the pattern of oxygenation and relative orientation of the aryl groups. Bryan and Fallon[42] confirmed by X-ray analysis the structure of syringaresinol as the 2,6-bis-(4-hydroxy-3,5-dimethoxy-phenyl)-3,7-dioxiabicyclo-(3,3,0)-octane.



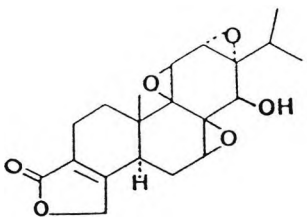
(23)
Syringaresinol

4.3.3 Terpenes

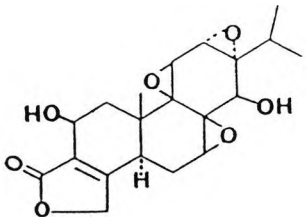
The report from Kupchan[41] in 1972 that an alcoholic extract of the plant possessed antileukemic and antitumour activities stimulated new interest. The alcoholic extract was found to show significant activity 'in vivo' against the L-1210 and P-388 leukemias in the mouse and 'in vitro' against cells derived from human carcinoma of the nasopharynx (KB).

Kupchan et al[41] reported the isolation and structural elucidation of triptolide (24) and tripdiolide (25), two novel antileukemic diterpenoid triepoxides. Fractionation of the alcohol extract, guided by assay against KB, L-1210 and P-388 revealed that the inhibitory activity was concentrated in the ethyl acetate layer of an ethyl acetate - water partition.[41] Elucidation of the structure and stereochemistry of the 3 diterpenoid triepoxide was based on spectral analysis and was confirmed by direct X-ray crystallographic analysis, conferring to triptolide, tripdiolide and triptonide the structures 24, 25, and 26, respectively.[41] These compounds and the companion cytotoxic ketone triptonide (26) were the first reported natural products containing the 18(4→3) abeo-abietane skeleton and the first recognized diterpenoid triepoxides. Triptolide, tripdiolide and triptonide were patented as antileukemic agents by Kupchan.[43-45]

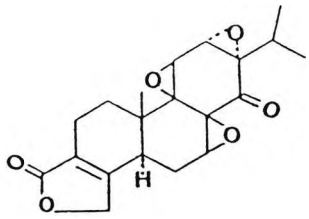
Chinese scientists[46] also reported the isolation of these terpenes from Tripterygium wilfordii. Triptolide and tripdiolide were also isolated from Tripterygium hypoglaucum.[47,48]



(24)
Triptolide



(25)
Tripdiolide



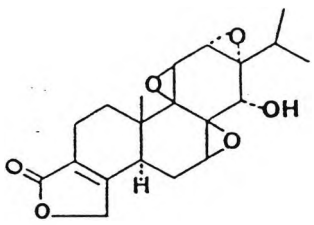
(26)
Triptonide

Potential antileukemic compounds often contain highly electrophilic functionalities and chemical and biochemical studies support the view that these compounds may act by selective alkylation of growth-regulatory biological macromolecules.[49] The selectivity may result from many factors, among which are transport of the tumor inhibitor into the cell and the chemical nature and steric environment of the specific nucleophile to be alkylated. Model studies support the hypothesis that the inhibition of tumor growth may be attributable to selective alkylation of nucleophilic groups in key enzymes which control cell division.[49]

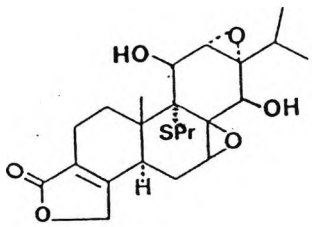
The diepoxide functionality has been shown to confer tumor-inhibitory activity on natural compounds, such as the natural occurring cyclohexane diepoxide, crotepoxide.[50] The α,β-unsaturated lactone function has been shown to be important for the tumor-inhibitory activity of several classes of terpenoids.[51]

The fact that triptolide (24) shows antileukemic activity in doses as low as 0.1 mg/kg while triptonide (26) shows no antileukemic activity in doses up to 0.4 mg/kg, led Kupchan et al to hypothesize that the 9,11-epoxy-14-hydroxy system present in triptolide but not in triptonide was necessary for the antileukemic activity of the triptolides. Futhermore, intramolecular catalysis by the 14-hydroxyl group may assist selective alkylation of biological macromolecules by the 9,11-epoxide. Subsequent testing of the minor variant

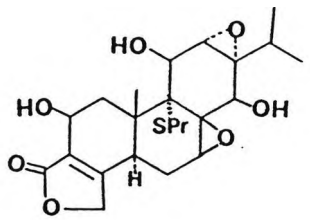
14-epitriptolide (27), with α-oriented 14-hydroxyl group, revealed that this triptolide derivative also shows no antileukemic activity at doses up to 0.4 mg/Kg. Thiol adducts from triptolide and tripdiolide (28) and (29), which lacked the 9,11-epoxide, did not show antileukemic activity at doses below 0.4 mg/Kg either.[52]



(27)
14-epitripdiolide

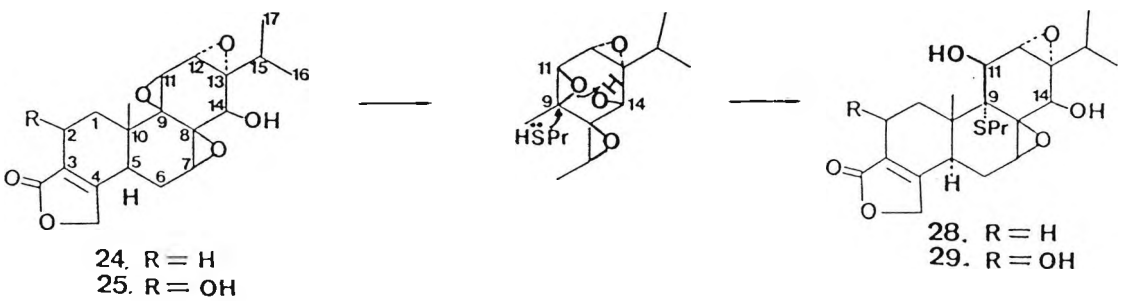


(28)
Triptolide adduct



(29)
Tripdiolide adduct

These adducts (28) and (29) were obtained by treatment of triptolide (24) and tripdiolide (25) respectively with a large excess of the nucleophile propanethiol, while on treatment of the variant 14-epitriptolide (27) with excess of propanethiol it was recovered unchanged. The increased reactivity of the antileukemic triptolide and tripdiolide relative to the inactive 14-epimeric derivative (27), was attributed to the 14-hydroxyl-group participating in the epoxy opening of the former compounds, as depicted in (30)(Scheme 4-1.)[21]



24. R = H
25. R = OH

28. R = H
29. R = OH

Scheme 4-1

The importance of the 9,11-epoxy-14-hydroxy system for the biological activity of triptolide and triptdiolide was also indicated by their relative cytotoxicities against KB

cells in culture. The median effective dose (ED_{50}) values for the series are (in mg/ml): triptolide (24) 0.0017; tripdiolide (25) 0.0042; triptonide (26) 0.021; epi-triptolide (27) 0.076; triptolide- 9-propanethiol adduct (28) >1, and tripdiolide-9-propanethiol adduct (29) >1.[52]

In 1981 Dujack compared the ultrastructural and cytotoxic effects of triptolide and tripdiolide, isolated from plant tissue culture, on normal and tumor cells. Bioassay studies revealed that the diterpene triepoxides inhibited the growth of 94% of the 48 species of bacteria tested, and were also biologically active against PYFR3T3 rat tumor cells. The treated cells became granular, rounded and detached from the substrate. A test conducted on the human tumor (KB) showed that it was inhibited by the supernatant and callous fraction from Tripterygium wilfordii tissue culture, at an ED_{50} of 3.7×10^{-1} µg/ml and 1.9 µg/ml, respectively.[53]

Triptolide (11) and tripdiolide (12) were also found to inhibit RNA and protein synthesis but these compounds had little effect upon DNA synthesis. An ultrastructural study revealed that the diterpenes triepoxides caused nucleolar aberration, resulting in a segregation of the fibrillar zone from the granular zone. However, triptolide and tripdiolide affected equally both normal and tumor cells.[53]

Triptolide was found to be toxic in mice and dogs, showing pathological or functional changes in the heart, liver and gastrointestinal tract. The haematopoietic system of the bone marrow was also depressed. The poison symptoms disappeared, however, after discontinuation of triptolide administration. No adverse effect was observed with a dose of triptolide lower than 20 µg/Kg per day.[54]

The antileukemic diterpenes are found in very low concentration (about 0.001%) in the Tripterygium wilfordii plant. Additionally, Tripterygium wilfordii is only indigeneous in some regions of China, it grows slowly and the roots are very difficult to collect. This encouraged a number of groups to develop synthetic approaches to the diterpenes,[55] two of which were successfully completed.[56-60]

A plant tissue culture could also conceivably alleviate supply problems, as it could be manipulated to accelerate the production process. Two main aspects in the research into tissue culture of Tripterygium wilfordii were: firstly, to determine whether the plant tissue culture would also produce biologically active compounds; secondly, to determine whether the yield of these compounds could be increased by optimizing the conditions of the growing media and by stimulating biosynthesis through precursor studies.

The need to measure the increased yield of tripdiolide and triptolide in the tissue culture media required the development of a rapid and reliable method to quantify the compounds without the interference of the tissue culture medium. Two analytical methods to determine tripdiolide had been previously published.[61,62] Both methods used HPLC, one with detection at 217 nm;[62] the other combined a C-18 column with a refractive index detector.[61] Unfortunately these analytical methods could not easily be applied to tissue culture as the high concentration of celastrol, an orange colour pigment of a quinone-methide structure which is strongly associated with the tissue culture assay, interferes with HPLC analysis and decomposition products of these quinone methide compounds bind irreversibly on all HPLC packing material.

The disposable nature of the TLC methods overcomes this interference problem. Early observations by Dailey and Kupchan[63] found that when TLC chromatograms of tripdiolide were sprayed with 2% ceric ammonium sulphate in 12% H_2SO_4 and heated on a hot plate until a brown spot became visible, the brown spot produced a blue fluorescence when viewed at 375 nm. Based on Kupchan's findings,[63] Kutney et al published in 1981 a quantitative method which was found to be linear for tripdiolide between 0.2 - 3.3 µg. The chromatograms were developed in methanol-chloroform on a silica plate with fluorescence indicator.[61]

A further TLC method was also reported using chloroform and ether as developing solvent, determining the content of triptolide by scanning the spots at 535 and 650nm. Applying this analytical method, it was found that roots from Tripterygium wilfordii harvested during spring and autumn contained 0.001 and 0.018 % of triptolide respectively.[64]

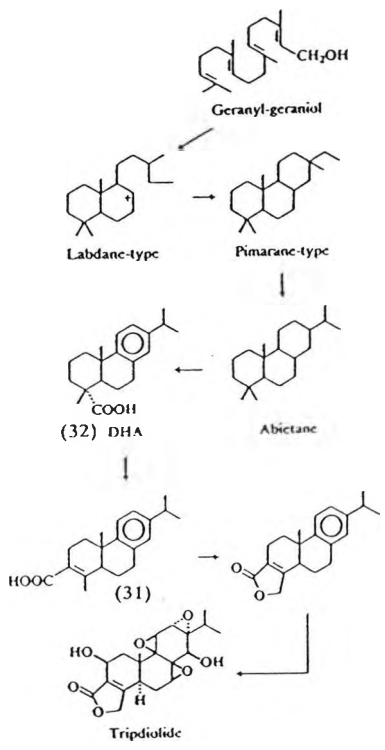
Successful production of secondary metabolites by plant cell tissue culture is frequently associated with cellular differentiation or organogenesis. Formation of aggregates, roots or shoots may involve development of specialized groups of cells which coordinate their metabolic activities to produce the secondary metabolite. Evidence suggests that Tripterygium wilfordii has this requirement for the production of good yield of tripdiolide.

The use of different media for stock culture maintenance and for production of secondary metabolites is common. Since the formation of secondary metabolites and growth are frequently inversely related, the media which supports product accumulation provides very slow growth.

In 1980 Kutney et al[65] developed a tissue culture method where the content of tripdiolide was increased threefold over that found in the plant. An improved method yielded 16-fold tripdiolide and 3-fold triptolide compared to amounts in the original plant.[66] Addition to the tissue culture growing medium of coconut milk, indole-3-acetic acid, vitamins and sources of C, N, Mg, Ca, P, S and K,[67] and the application of the continuous feeding tissue culture method by the use of a fermentator,[68] allowed Kutney et al[69,70] to obtain yields of tripdiolide 36 times greater than those reported for the plant by Kupchan.[44,45]

Kutney et al investigated the biosynthetic pathway leading to triptolide, hoping to finding precursors able to increase the yield of tripdiolide in the tissue culture technique.[66,69]

Scheme 4-2 outlines a possible biosynthetic pathway from geranylgeraniol to tripdiolide (25). The route from geranylgeraniol to the abietane skeleton has previously been reported.



Scheme 4-2: Biosynthetic pathway to tripdiolide

Kutney suggested that an important step in this pathway would involve rearrangement of dehydroabietic acid to a modified abietane skeleton, such as 15-hydroxy-18-norabieta -3,8,11,13,-tetraene-3-oic acid (31). Subsequent enzymatic oxidation would lead ultimately to triptolide (24) and tripdiolide (25) . The isolation by Kutney et al of dehydroabietic acid DHA (32) and the methyl ester of 15-hydroxy-18-norabieta-3,8,11,13,-tetraene-3-oic acid (31) from the tissue culture gave credibility to the above suggested pathway.[66]

Dujack et al[71] studied the influence of the presence of precursors in tissue culture on the yield of tripdiolide, finding that pyruvic acid and sodium acetate in the presence of light led to the highest yield of tripdiolide. Triptolide was present too.

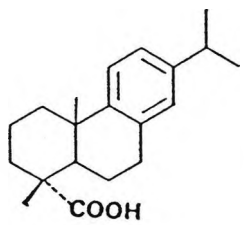
Misawa et al[72] obtained a 10-fold increased yield of tripdiolide when they added to the agar and sucrose from the tissue culture some phytohormones in order to improve the growth of the callous tissues.

All the above-mentioned scientists followed the method patented by Kupchan[43-45] to extract diterpene triepoxides from tissue culture. The method consists in an initial extraction with ethyl acetate followed by silica column chromatography to isolate the diterpene triepoxides from the crude extract.

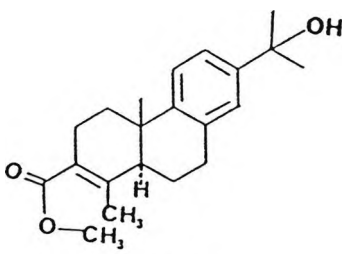
The Kyowa Hakko Kogyo Co.[73] in Japan submitted for patent in 1982 an improved tissue culture method to obtain tripdiolide in high yield. The isolation procedure included extraction of dry callouses from the tissue culture with 95% ethanol. The residue obtained after evaporation was dissolved in water and washed with hexane. Tripdiolide was finally

extracted with ethyl acetate and isolated by HPLC ODS-reverse phase chromatography. Further seven-fold improvement was achieved by supplementing the tissue culture medium with N-(2-chloro-4-pyridyl)-N-phenylurea.[74]

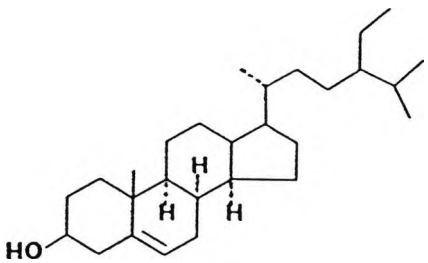
Other diterpenes have been also isolated from the tissue culture such as: dehydroabietic acid DHA (32),[66] 15-hydroxy-18-norabieta-3,8,11,13-tetraene-3-oic acid methyl ester (33),[66] and triterpenes such as β-sitosterol (34),[45,65,66] oleanolic acid (35),[66] populnonic acid (36),[66] and the triterpene pigment celastrol (37).[65,67]



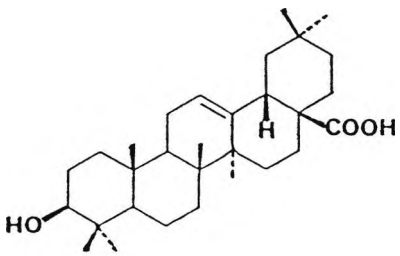
(32)
Dehydroabietic acid



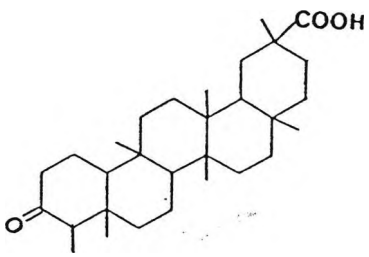
(33)



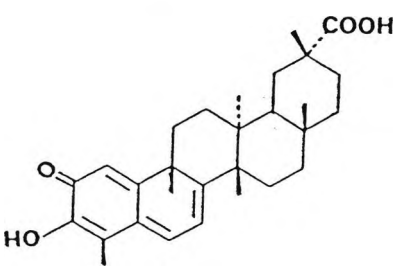
(34)
β-sitosterol



(35)
Oleanolic acid



(36)
Populnoic acid



(37)
Celastrol

In the early phytochemical studies on Tripterygium wilfordii, an insecticidal inert red pigment was isolated from the petroleum ether extract and it was named tripterine by the Chinese.[2] Schechter and Haller[8] found tripterine to be identical with the red pigment, celastrol, that Gisbold[75] had previously isolated from Celastrus scandens, the common

American bittersweet. Later, Nakanishi[76] amongst other scientists elucidated the structure of tripterine (37) based on spectral studies.

Celastrol or tripterine is the prototype compound of triterpene pigments, hence this group is designated under the name celastroid pigments. These numerous orange-red bark pigments found in the Celastracea family plants involve pentacyclic triterpene compounds with a glutinan skeleton. They have in common a conjugated 10 π-system with quinone-methide part on rings A and B. All the bonds in this conjugated system tend to perform a catalytic protonation rearrangement , where ring A and also ring B aromatize to the corresponding phenols. The tendency of the A ring of these quinoid structures to reduce easily has been documented.[77,78]

Tripterine was found by Zhang et al[79] to inhibit the proliferation of lymph cells.

In the course of studying the active principles of Tripterygium wilfordii, Qin et al[80,81] discovered in 1982 two new oleaneane-type triterpene lactones, wilforlide A and wilforlide B, in the roots of the plant. Based on the spectral data wilforlide A (38) was considered to be the γ-lactone of 3β,22α-dihydroxy-Δ^{12}-olene-29-oic acid. Wilforlide B (39) was proven to be the C3-dehydro-derivative of wilforlide A.[81] It is interesting to note that the γ-lactones of wilforlide A and B are formed only after the equatorial carboxyl and hydroxyl groups in ring E of the normal chair form have been converted into diaxial conformation in an unusual boat conformation of ring E (Fig. 4-2).

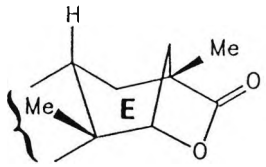
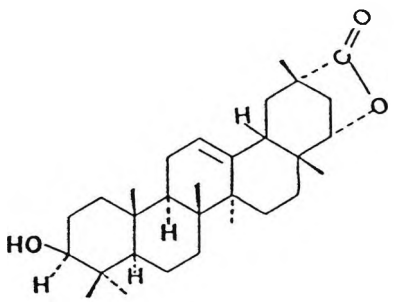
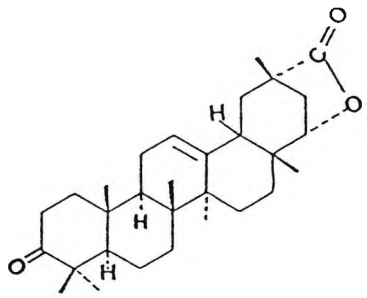


Figure 4-2



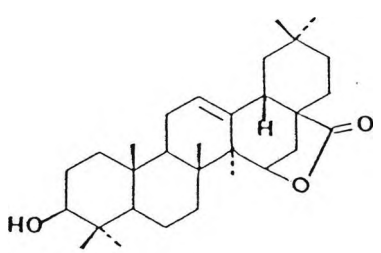
(38)
Wilforlide A



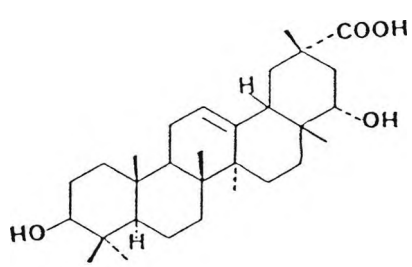
(39)
Wilforlide B

In 1974, Li et al[82] published the discovery of triptotriterpenoidal lactone A (40), a pentacyclic oleaneane type triterpenoidal lactone, whose structure was elucidated by spectral analysis.

Zhang et al[83] isolated in 1984 a new oleanane type triterpenic acid from the root of Tripterygium wilfordii and called it triptotriterpenic acid A (41), whose structure was finally elucidated by spectral studies. It was found to be effective as an anti-inflammatory agent.[83,84]

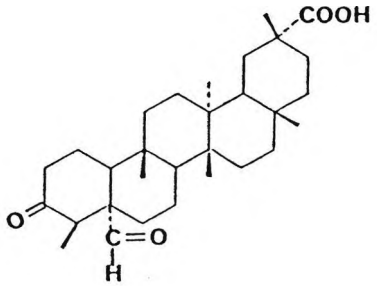


(40)
Triptotriterpenoidal
lactone A

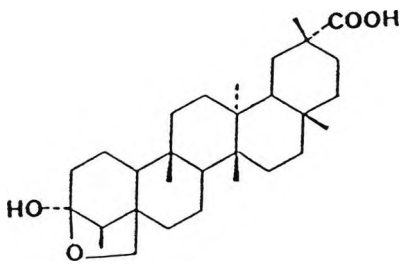


(41)
Triptotriterpenic
acid A

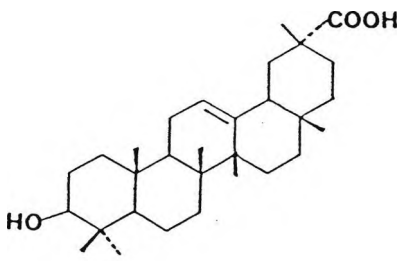
In 1986 Zhang et al[85] isolated 7 oleane type pentacyclic triterpenes from the root of Tripterygium wilfordii, and identified them as wilforlide A (38), wilforlide B (39), 3,24-dioxofriedelan-oic acid (42), salaspermic acid (43), 3-epikatonic acid (44), and also celastrol (37).



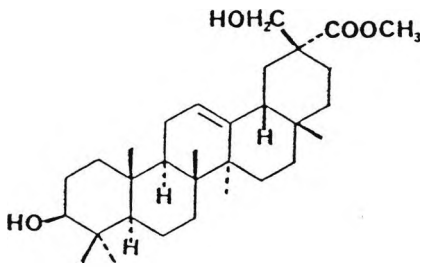
(42)
3,24-dioxofriedelanoic acid



(43)
Salaspermic acid



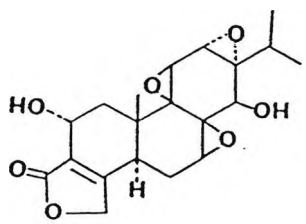
(44)
3-epicatonic acid



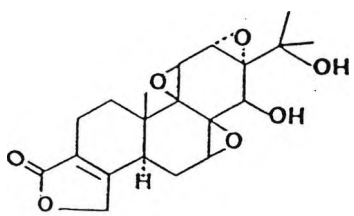
(45)
Triptodihydroxy acid methyl ether

A new triterpene: triptodihydroxy acid methyl ester (45) was isolated by Deng et al[86] in 1987 from Tripterygium wilfordii and identified by spectral studies.

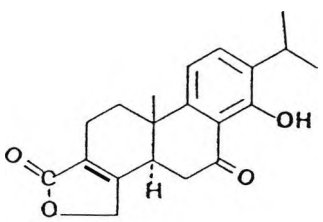
Also during the continuing phytochemical studies new diterpene triepoxides such as tripterolide (46)[87] and triptolidenol (47)[88] were isolated and identified. Many aromatic diterpenes were also isolated and identified by spectral studies: triptonolide (48);[89-93] hypolide (49) also named triptophenolide;[87,92-95] hypolide methyl ether (50);[66,70,95] neotriptophenolide(51);[95] isoneotriptophenolide (52);[92] triptonoterpene (53);[88,93] triptonorterpene methyl ether (54);[88,92] triptonoterpenol (55).[96] The biosynthetic origin of the keto-lactone wilforonide (56) has not yet been established.[92]



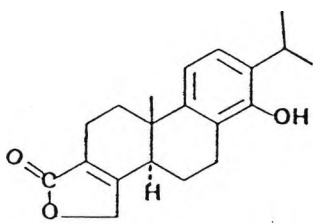
(46)
Tripterolide



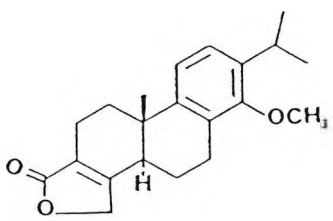
(47)
Triptolidenol



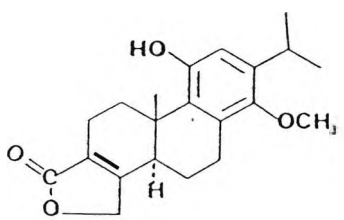
(48)
Triptonolide



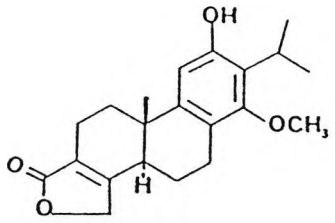
(49)
Hypolide



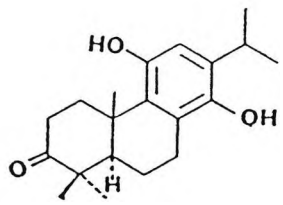
(50)
Hypolide methyl ether



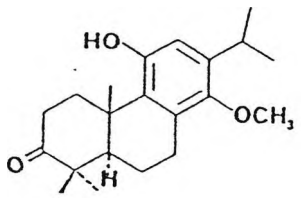
(51)
Neotriptophenolide



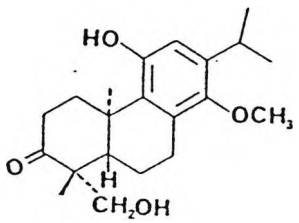
(52)
Isoneotriptophenolide



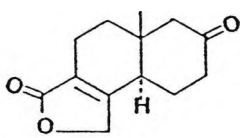
(53)
Triptonorterpene



(54)
Triptonorterpene
methyl ether



(55)
Triptonorterpenol



(56)
Wilforonide

4.4 Antifertility effects

The medicinal use of Tripterygium wilfordii in China can be traced back to more than two thousand years ago, when it was used for the treatment of fever, cold and edema.[97]

The preparation of a water soluble fraction from an initial alcoholic extract of the plant and subsequent extraction of the terpenes with ethyl acetate lead to a formulation of medicinal tablets.[90] These tablets contain, apart from the excipients, 20% of the extracted terpenes, including measurable amounts of triptolide, according to the Chinese.[90,98]

An alcoholic extract from the root of the plant has been used in the treatment of rheumatoid arthritis and ankylosing spondylitis with a therapeutic efficiency comparable to that of steroids. Serious side effects such as anorexia and stomach discomfort and menstrual disturbances were rather common. Mouth ulcers, pigmentation of the face, sore throat, conjunctivitis and mild hair loss usually cleared up after discontinuation or decrease of the dosage of treatment. Some patients showed pyrogenic and viral infections, which may be related to immunosuppressive activity of this drug, lowering the resistance of the patient. Similar therapeutic efficiency and side effects were observed by a different Chinese clinic, which prepared this drug by decoction and used an applied dose which was 10 times higher.[99]

The water-soluble fraction began to be referred to as a "total glycoside" extract of Tripterygium wilfordii in a publication by Zheng[100] in 1983, but no chemical evidence supporting the presence of glycosides, whether or not biologically active, has ever been reported.

In 1983 a less toxic formulation was prepared by removing the toxic alkaloids through extraction from the water-soluble extract. Further decoction of the plant followed by ethyl acetate extraction were the most common methods developed to produce a material which was called a glycoside fraction. The "glycoside fraction" was found to be therapeutically highly efficient in the treatment of rheumatoid arthritis. Side effects were still observed, including mild gastrointestinal disturbances, irregular menstruation and amenorrhea in female patients and gynecomastia in the male.[101]

Material termed "total glycosides" from the plant was also applied in the treatment of skin diseases, especially indicated in allergic diseases, autoimmune diseases and skin diseases with a mechanism possibly related to allergy.[102] The observed therapeutic effectiveness is claimed to be related to the anti-inflammatory and cellular and humoral immunosuppressive effects of the drug.[101,102] Anti-inflammatory properties of the extract have been substantiated by tests in animals,[103] and the toxicity effects in animals published.[100] Chinese scientists studied the effects on the reproductive organs of animals.[104,105]

In 1986 Qian et al[106-108] confirmed the antifertility effects in male rats by using a refined preparation of active fraction of Tripterygium wilfordii, described as GTW, short for "glycosides of Tripterygium wilfordii" following the unsubstantiated description by Zheng.[100] GTW, which is prepared by the Institute of Dermatology in Nanjing, China, is claimed, in reviews of unaccessible Chinese literature, to be prepared "by extracting the cuttings of the dried root xylem first with water, then with chloroform and finally GTW

are separated by column chromatography". 25 g of the xylem are calculated to yield 10 mg of the mixture, which is claimed to contain only minute amounts of diterpenes and alkaloids, the main toxic constituents of the plant. It is commercially available in tablets, each containing 10 mg of the extract.[101,102,109]

Qian et al. indicated that in male Wistar rats given GTW through gastric gavage at a dose of 10 mg/kg per day (5-10 fold the human dose calculated on the body weight basis), 6 times a week, the fertility of the rats began to decrease by the end of the fourth week of medication and at the end of the eighth week all the treated rats became infertile with a significant decrease in the density and particularly in the viability of the spermatozoa in the cauda epididymis, while the body weight growth, the mating behaviour, the blood testosterone level, the histology of the heart, lung, liver, spleen, small intestine, pituitary and hypothalamus, and the weights of the testis, ventral prostate and seminal vesicles were not significantly different from those of the controls.[106,108]

Qian et al[110] obtained similar results in male Sprague Dawley rats receiving GTW at the dose mentioned above. They also observed similar effects at a lower dose of 20 mg/kg per day in adult male BALB/c mice.[111]

In investigation of the effect of GTW on female mice by Qian et al[106] at a dose up to 50 mg/kg per day, GTW did not have anti-implantation effect, nor did it have early pregnancy termination effect. It was observed that, following oral administration of GTW at a dose of 30 mg/kg per day for 1 to 3 months to female mice[89] and rats,[83] the estrual cycle became irregular in 80-90% of the animals (ovarian function

was normal), there was decrease in weight of the uterus and slight change in the endometrium, although plasma estradiol and progesterone levels were normal. Compared to the toxic effects of the "glycosides" on male organs, female reproductive organs were much less affected: it was reported that the reproductive organs and fertility of female animals and the reproductive organs of the first 3 generations of their male offspring were not affected by GTW.[112] Further pharmacological effects,[103] antifertility effects,[104] and toxicity[100] in animals treated with GTW were described.

Observation on the anti-fertility side effects on male patients treated with GTW to cure skin diseases and several rheumatoid complaints revealed that a decrease in testicular volume may occur in some patients after a long-term treatment. Semen examination in treated patients showed that both the density and the motility of the ejaculated spermatozoa were far lower than in the non-treated subjects.[101] In some male patients taking only crude extract or the crude extract plus GTW for long term treatment, necrospermia or azoospermia occurred, recovering in most cases 3 months after cessation of treatment. In all patients libido, potency and the serum level of testosterone and LH were normal, while serum FSH was significantly higher in the treated than in the control group, but it was normal in the recovery group.[101,107,108] This side effect could be minimized if the dose of GTW was drastically decreased from the regular dose of 60-90 mg/day.

In an attempt to decrease the side effects of the other antispermatogenic agent gossypol, Xu et al.[102,113] administered sub-effective doses of gossypol and GTW simultaneously to rats to see whether their effects on male

reproduction were additive. Adult male SD rats were given, through gastric gavage, 5 mg each of gossypol and of GTW, 6 times a week for eleven weeks. Groups taking the vehicle and the same dose of GTW or gossypol alone were set as the controls. At the end of the 11th week, all the rats of the GTW-gossypol group became infertile as assessed by sequential fertility tests, while mating behaviour was present. The density and motility of spermatozoa in the cauda epididymis were dramatically decreased while no perceptible changes were seen in the Leydig cells, Sertoli cells and in most cases in the seminiferous and epididymal epithelia. All other parameters examined, including body weight growth, serum testosterone and the histology of various organs were not significantly different from the vehicle control. Both the fertility and the epididymal spermatozoa were completely restored 6 weeks after cessation of treatment. In the groups that took vehicle, only gossypol or only GTW , the fertility and the epididymal spermatozoa were normal. The results indicate that the effects of GTW and gossypol on the fertility of male rats are additive and reversible, which may provide a potential approach to the alleviation of the side effects of both antifertility drugs.[102,113]

Liu et al[114] published in 1987 their observation that Tripterygium wilfordii showed spermicidal activity to rat and human sperm.

Zhu et al[115] published in 1987 their study on the effects of 'total glycosides' of Tripterygium wilfordii on 'HeLa' tumor cells; the "in vitro" growth and division of

'HeLa' cells were inhibited and DNA synthesis by the tumor cells was also inhibited, whereas lactate dehydrogenase activity was not affected.

In 1987 Zheng et al[116,117] isolated various chemically undefined compounds termed "glycosides" from Tripterygium wilfordii and found that their antifertility activity seemed inseparable from their anti-inflammatory and immunosuppressant activities, although it appears that anti-inflammatory activity is separable from immuno-suppressant activity.

4.5 Background: Isolation and structural elucidation of male antifertility agents from Tripterygium wilfordii

4.5.1 Introduction

The reported low side effects of the Tripterygium wilfordii tablets at the relative low dose needed for reversible antifertility action in the male, encouraged further research into the active antifertility compounds in the plant.

In 1987, the WHO commissioned Professor Matlin's group at the City university to isolate all the active constituents of GTW tablets as confirmed by their antifertility tests in rats. Preliminary work on this project was carried out by V. Stacey and J. W. Zhang on four batches of TW tablets. The antifertility tests in rats were done by Xu Ye from the medical institute in Nanjing.

4.5.2 Strategy

The approach adopted by Professor Matlin's group in the present work was to make no initial assumptions about the number or chemical nature of the active male antifertility compounds which might be present in the materials available,

but to proceed by a systematic, stepwise fractionation process which would be bioassay-directed at every stage, so that all active components present would be identified. Allowing the possibility that active components (such as glycosides) might be sensitive to hydrolysis, it was first necessary to establish that tablets of proven activity could be dissolved in water and the solution freeze-dried without loss of activity. Once this was successfully demonstrated, a stepwise procedure was evolved which included preliminary solvent partitioning followed by successive chromatographic sub-fractionations. At each new stage, solvent extracts or chromatographic subfractions were submitted to bioassay: each active subfraction was then further subdivided and re-tested until the activity could be attributed to individual compounds whose structures were then determined.

HPLC was chosen to carry out the chromatographic subfractionation of the active solvent fractions, in preference to classical column chromatography or preparative TLC, due to its much higher resolution to separate a multicomponent mixture. Also, HPLC offers a fast separation and the eluting fractions can be easily monitored by measuring their UV, allowing reproducible subfractionation to be achieved.

The initial step was to chose a stationary phase which would elute all components, i.e. obtaining total recovery. Although an isocratic elution would be the preferred mode, the wide range of polarities of the components of each active solvent fraction would require the use of some gradient elution. In order to rapidly locate the active ingredient, the active solvent fraction would be initially run using a multiple step gradient mobile phase. Later when

activity had been allocated to a specific fraction, a continuous gradient elution should be applied emphasizing resolution in the polarity range where activity was found, while maintaining a short run time. Those subfractions in which activity was allocated would have to be further chromatographed for further screeening of antifertility activity of single components. These components should be pure enough for proper structural elucidation.

Once the antifertility components of TW tablets had been identified and chemical structures assigned, a more efficient tactic could be used to isolate and purify them large scale. The new separation tactic could involve initial enrichment of the active ingredient from the active solvent fraction by either preparative TLC, classical column chromatography or flash chromatography. The enriched active fractions could be then easily subfractionated by the previously developed HPLC methods.

4.5.3 Materials

4.5.3.1 Sources of Tripterygium wilfordii

Supply of a single large batch of GTW tablets from China to carry out the project proved to be very difficult. Instead various smaller batches of GTW tablets were received from China and were coded in chronological order on their arrival (Table 4-1).

Code	no of tablets	no test equivalent
TW100	300	1.66
TW200	1000	5.55
TW300	2000	11.1
TW400	44500	80.3

Table 4-1

The average tablet weight of the different batches of GTW tablets ranged between 70.7 and 78.5 mgs.

One test equivalent (180 tablets) of each of these batches were tested on rats before fractionation and all of them proved to be active.

A large batch (500 g) of crude TW extract was promised by the Chinese authorities as part of a cooperative agreement with WHO: in 1987 ca. 200 g of powdered material was acquired prepared by extraction of T.wilfordii. This material was coded as Series 500.

Later (1988), 50 g of another crude extract were received, which was coded as Series 600. Most recently, a sample of 85 g plant extract has been received, which has been coded as Series 700.

In each case, it was said that these extracts correspond to the same material that would be directly incorporated into TW tablets. However, no details of the extraction or refining processes were provided with the materials.

4.5.3.2 Reference compounds

As detailed in the review of TW literature (Chapter 4.3), numerous compounds in the TW plant, such as diterpenes (anti-tumour agents) have been isolated and identified. Dr. Jim Kutney at the University of British Columbia, who has worked extensively on the development of plant tissue culture methods for producing TW diterpenes to be used in the US National Cancer Institute studies, supplied small samples of tripdiolide and later triptolide, on request from Dr. Matlin.

4.5.4 Extraction

TW tablets were initially ground to powder and subjected to various extraction procedures (Scheme 4-3 and 4-4). The general extraction procedure is shown in Scheme 4-3, with code numbers exemplified for Series 200 (note that coding is consistently assigned in all Series so that the first digit denotes the Series, the second is usually 0 but otherwise indicates a repeat run and the third digit represents the solvent fraction type). The tablets were dissolved in water, filtered and, if the whole material was to be tested, freeze dried for shipping. To examine the stability to hydrolysis, a batch of whole aqueous extract was boiled with acid before neutralization and freeze drying (Scheme 4-3,C). For fractionation, in the first two series of experiments (Scheme 4-3, B) the filtered aqueous solution was extracted with ether (pH 8.5) to remove neutral and non-polar components, ethyl acetate at pH 3.0 to remove more polar and/or acidic components, then neutralized and freeze dried. In the third and fourth series of experiments (Schemes 4-4, C), the aqueous solution, after ethyl acetate extraction at pH 3, was further extracted with n-butanol, which should extract glycosides of materials such as terpenoids, before neutralization and freeze drying. The possibility of extracting the active components from the final freeze-dried aqueous residue using methanol was also explored (Scheme 4-4, C).

The weights of material obtained per tablet from three series of extractions on different batches of tablets are shown in Table 4-2. There is significant variation between tablet batches in the content of organic-soluble components of each fraction.

A. Tablets —(Aq. soln; Filter, Freeze dry)→ Solid residue 201*

B. Tablets —(Aq. soln; Filter, OH^-)→ pH 8.5 —→ Et_2O phase —(Dry)→ Solid 204

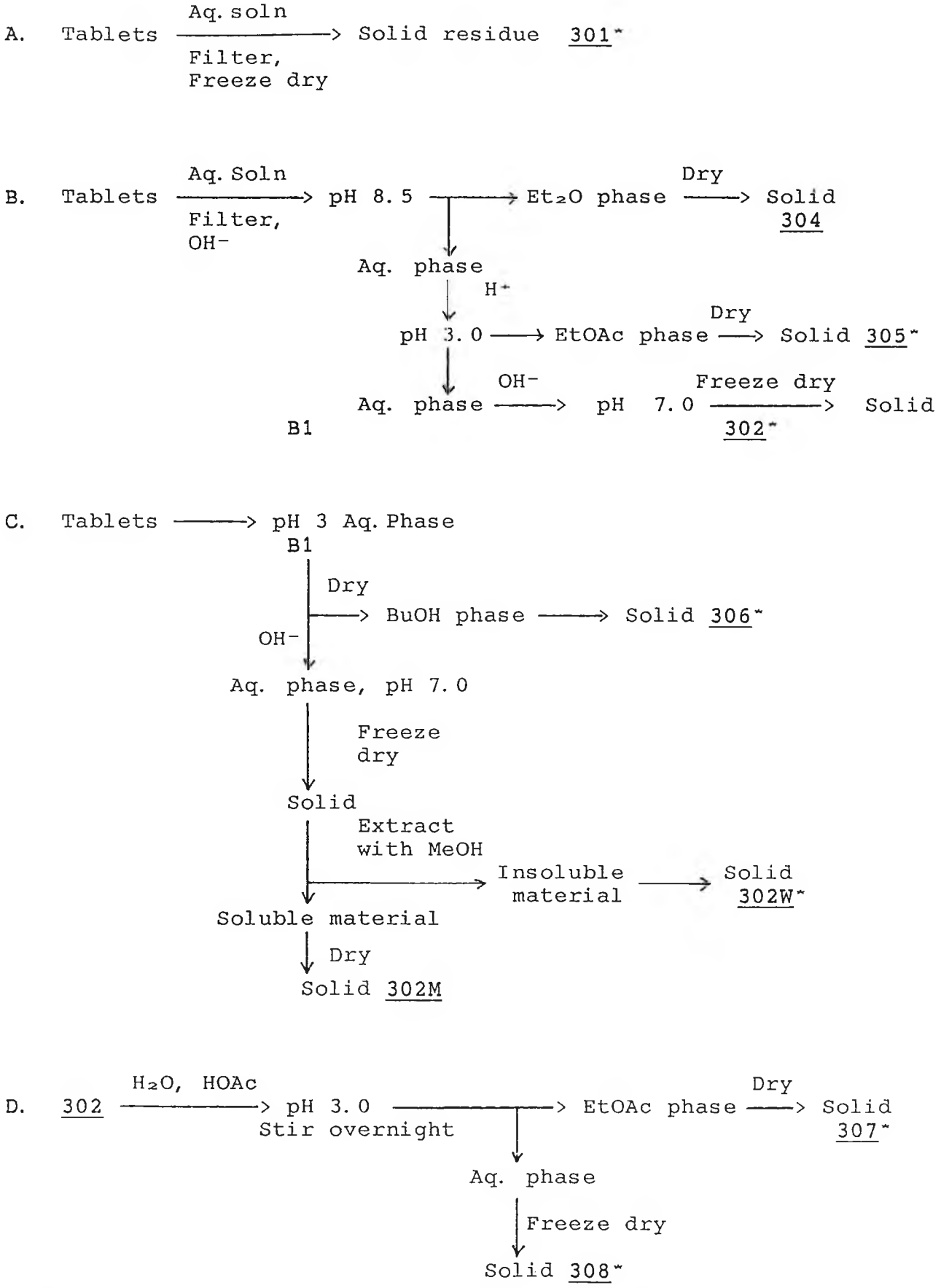
pH 8.5 → Aq. phase —(H^+)→ pH 3.0 —→ EtOAc phase —(Dry)→ Solid 205*

pH 3.0 → Aq. phase —(OH^-)→ pH 7.0 —(Freeze dry)→ Solid 202*

C. Tablets —(Aq. soln; H^+ to pH 1)→ Boil 3 hrs —(OH^-)→ pH 7.0 —(Freeze dry)→ Solid 203

* has partial or total male antifertility activity in rats.

Scheme 4-3 Extraction sequence for TW extract Series 200 (identical sequence used for TW extract Series 100)



* has partial or total male antifertility activity in rats.

Scheme 4-4 Extraction sequence for TW extract Series 300 (identical sequence used for TW extract Series 400)

Table 4-2. Recovery of material following extraction of tablets of Tripterygium wilfordii.
Quantities are expressed in mg/tablet.
Extraction methods are shown in Scheme 4-3 and 4-4.

Extraction Series	Whole Aqueous Extract	H+-Boiled whole Aqueous Extract	Et_2O Extract	EtOAc Extract	Extrac. Aqueous Residue	Extrac. Aqueous Residue/ /MeOH Soluble	Extrac. Aqueous Extract MeOH Insol.	BuOH Soluble Extract Aqueous Residue	Aqueous Extract pH3(HOAc) /EtOAc Phase	Aqueous Extract pH3(HOAc) /Aqueous Residue
TW-100	101 9.2	103 20.8	104 0.75	105 2.9	102 10.1					
TW-200	201 18.2	203 22.4	204 0.06	205 0.42	202 21.6					
TW-300	301 18.9		304 0.14	305 0.5	302 18.6	302M 0.004	302W 16.3	306 0.24	307 0.075	308 18.6
TW-400			404 0.20	405 0.32	402 14.5				407 0.01	408 14.12

4.6 Results:

4.6.1 Solvent fractionation and testing in rats

Series TW 100

Initially (prior to full information being available on the quantities necessary for bioassay), this batch of tablets was sub-divided into 3 equal lots and a trial extraction protocol developed as shown in Scheme 4-3. Thus, about 60 tablets were used in each of the protocols A, B and C and initially the fractions obtained were therefore not tested, but were kept for reference and later study (see below).

Series TW 200

A lot of 180 tablets and the whole, freeze-dried aqueous residue from a further lot of 180 tablets were sent to Dr. Bialy at NIH for confirmatory bioassay. Of the remainder, one lot of the whole, freeze-dried aqueous residue from 180 tablets (TW201), and extracts prepared from 180 tablets according to Scheme 4-3 (TW202-205) were sent to Dr. Qian for bioassay.

The bioassay results from Dr. Qian's laboratory are shown in Table 4-3. Several very important facts emerge from these results:

1. Antifertility activity in the total tablet extract 201 is confirmed.
2. This activity is entirely lost on vigorous acid hydrolysis: 203.
3. An active antifertility component is extracted into the ethyl acetate phase 205.
4. An active antifertility component remains dissolved in the aqueous phase after ethyl acetate extraction: 202.

5. A partial reduction in sperm count and proportion of live sperm of feeding the ether fraction suggests that a sub-threshold dose of an antifertility agent might be present in this fraction 204 and required further exploration (see Series 300 below).

At this point, it was recognized that, although Series 100 fractions had been obtained from only ca 60 tablets rather than 180, the actual weight of ethyl acetate (105) and ether (104) fractions were higher than in the 200 Series (Table 4-2). It was therefore recommended to Dr. Qian that the fractions from Series 100 be tested: this was done, using 2 rats/fractions. Despite the small number of tablets and small test group, the results were fully consistent with those obtained in Series 200 (Table 4-3).

Table 4-3
The effects ofTW extracts Series 100 & 200 on fertility of male rats.+

Extract code / (no. rats - test)	Body weight gain (g)	Weigth of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml	Female rats cohabited with males no @ rats	Female rats cohabited with males preg-nant	Female rats cohabited with males Fetuses/ preg. rat	
Ctrol(2)	111±14	4.00±0.16	83±4	59±17	4	4	13.2±2.4	
101(2)	112±11	2.53±0.11	2.5±4.2	19±27	2	0	0	A
102(2)	103±45	4.14±0.12	0	34±9	2	0	0	A
103(2)	119±0	4.17±0.4	82±5	47±13	2	2	10±2.8	
104(2)	99±35	3.73±0.17	75±7	32±8	2	2	12±2.3	
105(2)	69±6	4.81±0.3	0	21±9	2	0	0	A
Ctrol(5)	108±28	3.76±0.3	85±3.5	71±12	5	5	12.8±1.6	
201(4)	95±12	2.63±1.0	0**	7.3±7.2	4	2	7.0±5.6	A
202(5)	80±36	4.2±0.2	0.1±0.2	16.9±7	5	0	0	A
203(5)	90±36	4.4±0.6	65±21.1	67.2±17	5	4	9.5±2.1	
204(4)	91±19	3.9±0.3	57.5±7	33.6±25	4	4	10.8±1.0	
205(4)	61±29	5.0±0.7	0.15±0.1	15.5±11	4	1	11	A

+ Data expressed in x±SD, if applicable

@ One male rat is caged with 2 females. When spermatozoa are found in found in the vaginal smear of one rat, both females are removed and the one with the positive smear is sacrified on day 16.

**Examination of epididymal spermatozoa was done one week after cohabitation. Therefore, it is considered that live sperms were present during cohabitation

A Active fraction

Series TW 300

A total aqueous soluble extract of 180 tablets was freeze-dried to reconfirm the antifertility activity. Various extracts of the remaining 1620 tablets were then prepared according to Scheme 4-4: the ether (304) and ethyl acetate (305) extracts as before, to generate large quantities of material for isolation: BuOH and MeOH treatment of the final aqueous portion and its freeze-dried residue were carried out to begin to explore ways of isolating the most water-soluble active compounds.

Series 300 fractions were sent to Nanjing for testing and the bioassay results from Dr. Qian's laboratory (Table 4-4) led to several important conclusions:

1. Antifertility activity in the total extract (301) was again confirmed and consistency was also seen in the extraction processes: an active antifertility component was extracted into the ethyl acetate phase (Fraction 305), whilst a further active antifertility component remained dissolved in the aqueous phase (Fraction 302) after ethyl acetate extraction.
2. A slight reduction in sperm count and proportion of motile sperm on feeding the ether fraction was initially observed (Fraction 204): further testing (Fraction 304) showed that there is no significant activity in the ether extract and work on this fraction was terminated.
3. When the active aqueous residue (Fraction 302) was partitioned between water and butanol, strong activity was observed in both fractions (fraction 306 and 302W): This may indicate either that (i) there are two different active components in the aqueous residue, with different solubilities, or that (ii) there is one active

compound present in significant amount and partitioning fairly equally between the two phases. Both explanations are consistent with the behaviour of glycosides and other hydrophilic compounds.

4. Since it has been shown earlier (sample 103 and 203), (table 4-3) that vigorous acid hydrolysis led to loss of activity in the TW extracts, a further experiment was carried out to examine the effects of mild acid hydrolysis (Scheme 4-4,D). The aqueous solution formed by dissolving 180 tablets of Series 300 was adjusted to pH 3 with acetic acid and the solution stirred overnight at room temperature. Following the usual extraction protocol then led to the isolation of the ethyl acetate soluble fraction (307) and neutralized freeze dried aqueous residue (308). Bioassay (Table 4-4) showed that activity had been retained in both these fractions following the exposure to mild acid.

Table 4-4
The effects ofTW extracts Series 300 on fertility of male rats. +

Extract code / (no. rats - test)	Body weight gain (g)	Weigth of testis (g/kg)	Epididymal sperm		Female rats cohabited with males			
			% live sperm	Density 10^6/ml	no rats	preg-nant	Fetuses/ preg. rat	
Ctrol(5)	125±11	4.00±0.5	78±2.7	64.5±43	5	5	13.4±1.5	
301(5)	108±11	3.3±0.8	0	5.2±3.7	5	1	11.3±0	A
302(5)	111±20	3.9±0.3	0.8±1.1	16.2±7.9	5	1	16.0	A
302W(5)	112±13	4.2±0.5	2.4±0.5	31±7.1	5	0	0	A
304(5)	127±27	3.8±0.7	66±22	70.1±28.	5	5	12.2±0.8	
305(5)	121±25	4.1±0.5	1.0±2.2	31±11.7	5	1	12.0±0	A
306(5)	144±15	3.8±0.1	9±17.5	34±26.6	5	1	10.0±0	A
Ctrol(5)	109±16	4.0±0.2	82±4	52±3	5	5	12.8±2.4	
307(5)	86±13	4.3±0.5	30±10	18±5	5	2	11.5±0.7	A
308(5)	87±12	4.5±0.5	3±3	17±8	5	0	0	A

+ Data expressed in x±SD, if applicable.
A Active fraction

Series 400

The series 400 tablets were extracted using the same protocol (Scheme 4-4): it was confirmed (Table 4-5) that the whole, water soluble, filtered, freeze-dried contents (400) were active, as were the EtOAc fraction (405) and aqueous residue (402) from these tablets. These extracts therefore provided the first large pool of material for purification. Dr. Qian informed Professor Matlin's group that bioassay of the Series 400 tablets indicated apparent potency level twice that of the earlier Series.

In addition, with the much larger quantity processed there was a significant amount of solid material (406) recovered from the initial filtration after dissolution of the tablets in water and found to be active on bioassay (Table 4-6). 406 proved to be freely soluble in ethyl acetate and analytical HPLC indicated that the bulk of the sample was very non-polar (plant lipids?).

Table 4-5
The effects ofTW extract Series 400 on fertility of male rats. +

Extract code / (no. rats - test)	Body weight gain (g)	Weigth of testis (g/kg)	Epididymal sperm		Female rats cohabited with males			
			% live sperm	Density 10^6/ml	no rats	preg-nant	Fetuses/ preg. rat	
Ctrol(5)	109±16	4.00±0.2	82±4	52±3	5	5	12.8±2.4	
402(5)	108±16	4.4±0.4	3±5	8±5	5	0	0	A
405(5)	109±20	3.8±1.2	18±21	14±8	5	1	1	A
407(5)	184±42	1.5±0.1	5±1	18±9	5	0	0	A
408(5)	121±40	1.5±0.1	4±2	23±18	5	0	0	A
Ctrol(10)	191±20	3.44±0.25	79±5	68±16	10	10	12.6±3.1	
400(10)$	185±28	3.29±0.33	0	22±7	10	0	0	A

+ Data expressed in x±SD, if applicable.
A Active fraction
$ Total tablets assayed. Dose had to be reduced to half: i.e. 5 mg/kg/day, due to high activity which caused testicular atrophy at 10 mg/kg/day.

Table 4-6
The effects of 406 on the fertility of male rats. +

Group(n)	Body wt gain (g)	Weight of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml
Control(5)	111±25	1.65±0.18	90±4	85±10
406 (5)	97±24	1.56±0.12	4.0±2.2*	18±10* A

+ Data expressed in x±SD, if applicable
A Active fraction
* P<0.001, in comparison with the control.

4.6.2 Testing requirements

In his preliminary antifertility work, Dr Qian had concluded that a treatment with 1 tablet/rat/day (equivalent to 30 mg/kg/day) for 30 days (actually dosed at 6 days/week for 35 days) efficiently suppressed fertility in male rats without undesirable side effects, such as reported earlier.[53,99,101] Thus, for a group of 6 male rats in a test group, the contents of 180 tablets are required for a single test over a period of 5 weeks. This dosing level was initially applied to all solvent extracts and preparative HPLC fractions, but after some observations of poor recovery from certain HPLC procedures (see below), a five-fold increase in dosing was decided upon for later testing of HPLC subfractions to avoid false negative results.

4.6.3 Fraction testing in rats

At the outset of the work, GTW was administered to SD rats through gastric intubation at a dose of 30 mg/kg per day, 6 times a week for 5 weeks. At the end of the 5th week two female rats were cohabited with each of the male rats. The males were sacrifized and the body weight, weight of the testes and density and motility of the epididymal sperm recorded versus a control group. Two weeks after cohabitation, the female rats were sacrificed and the

pregnancy rate and the number of viable and resorbing fetuses were recorded versus a control group. Initially, the antifertility potencies of the fractions were assessed as the rate of pregnancy of female rats cohabited with male treated for 5 weeks.

Reviewing the data after several rounds of testing, Dr Qian concluded that in all treated and control groups of rats there was a direct relationship between the motility or density of the epididymal spermatozoa and the pregnancy rate of the cohabited female rats at the end of the treatment. In a WHO Male Task Force meeting in Geneva in October of 1988, Dr. Qian persuaded the Steering Committee that in future he should assess the antifertility potency of the GTW fractions by means of the sharp decrease the motility and density of the epididymal spermatozoa of the male rats, after 5-6 weeks of treatment. This new approach would no longer require cohabitation of females rats for pregnancy tests, freeing many cages and therefore allowing an increased number of tests to be carried out. Additionally the new antifertility tests were speeded up by 2-3 weeks.

4.6.4 HPLC studies

4.6.4.1 Ethyl acetate fraction

Series 100 - 300

The test results for Fraction 105, 205, 305 (Table 4-3 and 4-4) showed the presence of one or more compounds with antifertility activity in this ethyl acetate extract.

At the outset, with no knowledge of the structure of the chemical entity being sought, there was a problem in selecting a suitable wavelength for UV detection on HPLC. Initially, playing a hunch, 235 nm was chosen for preliminary studies.

On HPLC examination of the ethyl acetate extracts of each Series, the cleanest looking preliminary separations were obtained on an aminopropyl silica column used in normal phase mode with single step gradient elution. There also seemed to be good consistency in qualitative composition between the batches, with two groups of compounds of widely differing polarities present, the more polar requiring the use of the gradient step. There is one component which appears as a well-resolved, discrete peak and in apparently greater concentration than any other ("apparently", because relative response factors at 235 nm are unknown) and located in the less polar region of the chromatogram. This component has a second, much smaller one running very close to it. The major peak was initially referred to as "Compound X" (Figure 4-3).

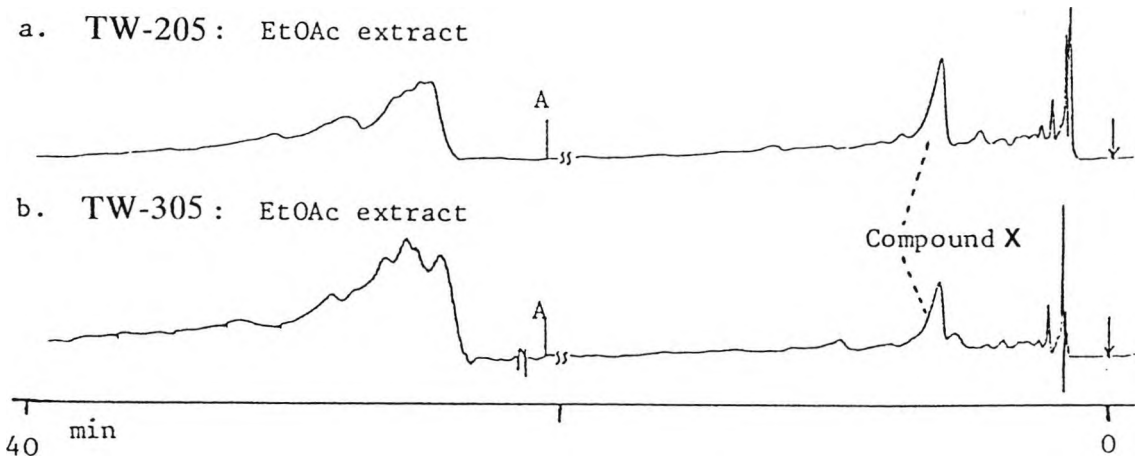


Figure 4-3. HPLC of solvent extraction fractions prepared from tablets of Tripterygium wilfordii according to Extraction Schemes 4-3 and 4-4. Column 25 x 0.45 cm Hypersil-APS 5 µm, eluted initially with 8:2:10:80 MeCN-iPrOH-Hexane-CH_2Cl_2, switching to 15:5:80 MeCN-iPrOH-CH_2Cl_2 at point A; monitored at 230 nm.

A large column (45 cm x 22 mm i.d.) was assembled and packed with a synthesized batch of Lichroprep-APS aminopropylated silica. Preparative HPLC on this column gave similar results to the analytical separations, but allowed

large quantities to be eluted and collected. For localization of the biological activity, the eluate was split into 4 section: everything before "Compound X" and its associated smaller peak (Fraction 305-1), "Compound X" and its associated peak (Fraction 305-2), the eluate between "Compound X" and the beginning of the gradient step (Fraction 305-3) and everything after the gradient step (Fraction 305-4). After evaporation of the eluate fractions and weighing, quantities of each fraction derived from the content of 180 tablets were conveyed to Nanjing for bioassay (October 1986).

As the (apparently) major single species present in the active ethyl acetate extract, "Compound X" attracted attention as being the active constituent. A sample of fraction 305-2 was re-purified by preparative HPLC for structural elucidation. The ^{1}H-NMR was very similar to the published data for triptolide.[43-45] No further spectra were done at this time and this compound was used as a reference compound in later HPLC separation.

Of the four fractions (305-1 to 305-4) generated by preparative HPLC on an aminopropyl silica (APS) column, only the 305-4 material appeared to be active from bioassay. However a serious problem encountered in this separation was that the material recovery from the preparative APS column was relatively poor (ca. 15%) and a lot of polar material was retained on the column. It seemed very likely, therefore, that active components might be lost entirely or reduced to a low level at which their activity would not be detected. In view of this, Professor Matlin's group decided to repeat the work with a reverse phase method on octadecyl silica (ODS). At the same time, they had an opportunity to incorporate a much more sophisticated detector system.

New HPLC system

Detector

In October 1986, Professor Matlin's group acquired a new Hewlett Packard photodiode array detector. This repeatedly scans all wavelengths in the UV/Visible spectrum (at a maximum rate of once every 10 microseconds) during the HPLC run and stores the data on hard discs, from where it can be recalled for processing. The wavelength domain is added as a third dimension to time and absorbance, without stopping the eluent flow. The chromatogram can then be viewed at any desired single or multiple wavelengths, UV spectra of components at any retention times can be viewed and compared and different HPLC runs can be overlaid for comparison.

Application of the new detector to TW fractions confirmed that 235 nm was the optimum for detection of all the components eluting: the UV spectra of all these components were recorded and filed.

New separation conditions

Because of the rather poor sample recovery of EtOAc extract components on APS noted above, and because the aqueous fraction contains even more polar components, Professor Matlin's group decided to change to reverse phase HPLC conditions for all further work. A new system was developed involving a step-gradient from aqueous methanol to a washing step of 100% acetonitrile on ODS silica. The various subfractions collected were all found to be inactive at 1 test equivalent. At this point I joined the project.

4.7 References

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CHAPTER 5

TRIPTERYGIUM WILFORDII: RESULTS AND DISCUSSION

5.1 Introduction

The previous work carried out on the liquid-liquid extraction of active ingredients of TW tablets, established activity in both the ethyl acetate phase and the aqueous phase, as shown in Tables 4-3 to 4-5 corresponding to Schemes 4-3 and 4-4. The fractionation of the ethyl acetate extract on a Hypersil-APS HPLC system had to be abandoned as the recovery was very low (20%). Subsequently, V. Stacey developed a reverse phase separation for the same fraction, TW305, on a silica-ODS system; no activity was found on any of the separated fractions recovered from "1 test equivalent" (i.e. the content of 180 tablets), while the starting material at the same test equivalent was active. The negative results could indicate that the fractionation of 1 test equivalent of the active fraction led to a loss of activity for the subfractions, bringing their remaining activity below the threshold level. It was therefore decided, at the start of my experimental work for this project, to run all preparative HPLC fractionation at 5 test equivalents, in the search for all components with antifertility activity in both the ethyl acetate and aqueous fractions. In order to obtain recoveries, close to total, alternative HPLC stationary phases and different chromatographic modes were investigated; these are described in the following sections.

5.2 Material used

This project was initially carried out on the material from Series 400. When all the material from the last tablet batch (TW400) was used up, fractionation was continued on the plant extract TW600, which had been tested and found to be very potent.

5.3 HPLC fractionation

ODS stationary phase

At this stage of research, it was found desirable to study the resolution of the components of TW by reverse phase chromatography on a non-polar stationary phase, such as the chemically bonded alkyl-silica ODS phase, for which semipreparative and preparative scale HPLC columns would be readily available.

From the outset, buffer solutions were avoided in the mobile phase, as possible association between buffer salts and some potentially active components might result in loss of their antifertility properties. Therefore, only solvents such as water, acetonitrile, methanol and tetrahydrofuran were considered in the development of the HPLC fractionation method.

For the ethyl acetate crude fraction, TW405, an isocratic MeOH/H_2O system provided some separation, although the eluting peaks were excessively broad. On the other hand, an MeCN/H_2O system eluted the components earlier and sharper, sacrificing some separation. For the aqueous fraction, TW402, a similar lack of resolution was found; all components eluted much earlier than the ethyl acetate fraction due to their increased polarity.

5.4 Fractionation of the ethyl acetate phase (TW405)

5.4.1 ODS step-gradient

To achieve a reasonable fractionation of the very complicated ethyl acetate extract of TW, gradient elution was investigated on an analytical scale. A 2 step gradient using acetonitrile, methanol and water was developed. The best compromise between sharp eluting peaks and improved resolution was obtained when an aqueous mobile phase was used containing methanol/ acetonitrile in a ratio 1:1 (Fig. 5-1a). Reproducibility of this chromatogram proved to be remarkably good.

These conditions were scaled up on 1" O.D. preparative column and a UV detector was fitted for this purpose with a 1 mm preparative flowcell. A loading of 100 mg could be achieved with reasonable separation and the recovery was calculated to be 75.5%; 3 subfractions were collected and coded as presented in Figure 5-1b.

Antifertility tests of 5 test equivalents (Table 5-1) demonstrated antifertility activity only in the middle fraction 415-2.

Table 5-1
The effects of 415 series on the fertility of male rats.+

Group(n)	Body wt gain (g)	Weight of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml	% w/w starting material	
Control(5)	157±31	1.58±0.04	84±4	85±13		
415-1(5)	153±12	1.63±0.05	62±14	62±13	26.7	
415-2(5)	125±14	1.54±0.08	7±5.7	27±10	40.4	A
415-3(5)	157±17	1.63±0.12	81±7	87±20	32.8	

\+ Data expressed in x±SD, if applicable
A Active fraction

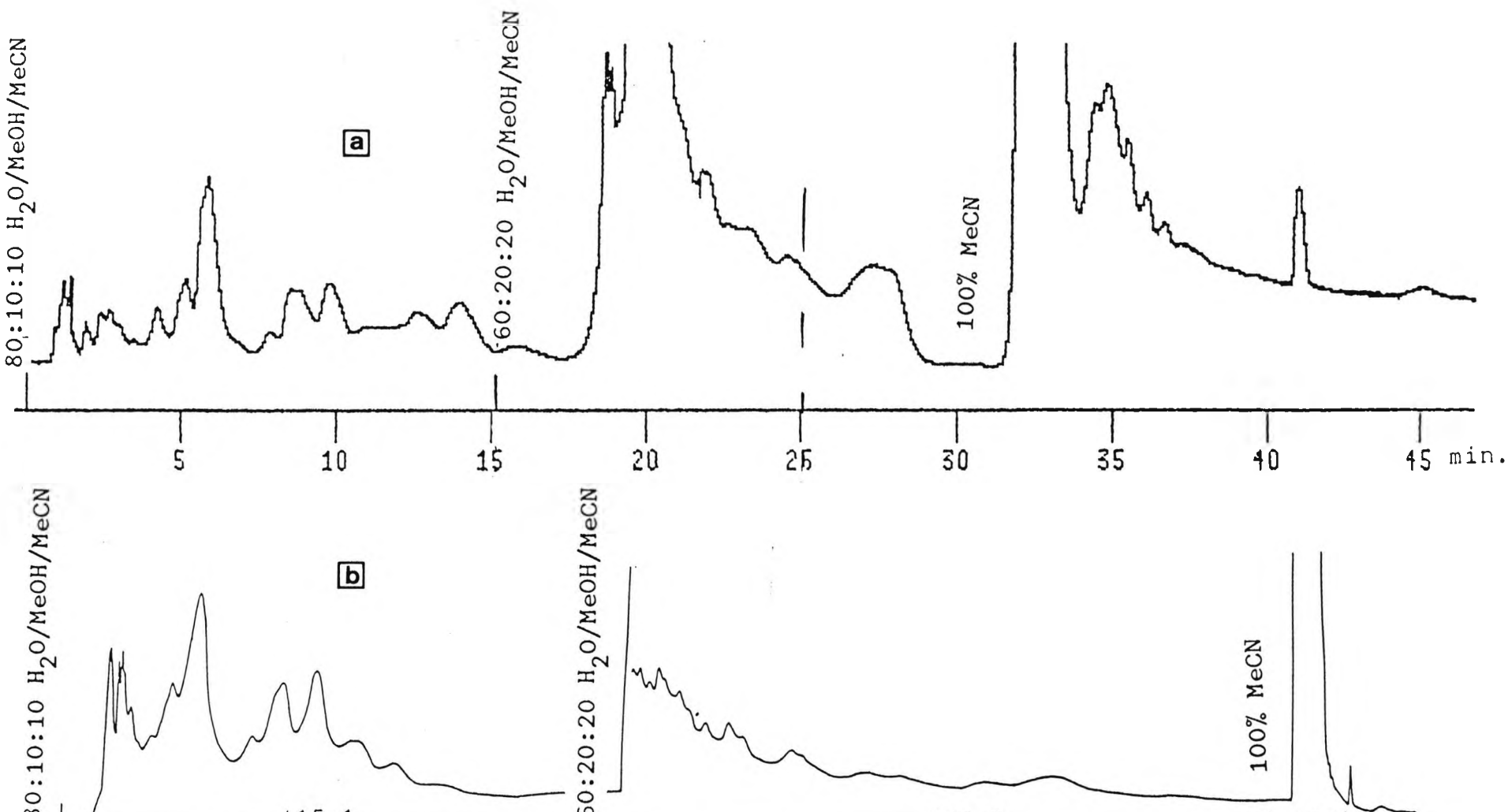


Figure 5-1. Multistep gradient HPLC separation of TW405 on ODS. a) Analytical HPLC on 25 x 0.45 cm ODS-Hypersil 5 µm eluted with 10:10:80 MeCN/MeOH/H_2O from 0-15 min., 20:20:60 MeCN/MeOH/H_2O from 15-25 min. and 100% MeCN from 25-45 min. at 2 ml/min, monitored at 230 nm. b) Preparative separation on 1" O.D. x 33 cm Hypersil-ODS 5 µm, eluted with 10:10:80 MeCN/MeOH/H_2O from 0-35 min., 20:20:60 MeCN/MeOH/H_2O from 35-85 min., and 100% MeCN from 85-95 min., at 30 ml/min, monitored at 230 nm, at 0.05 AUFS, loading 200 mg in a 4 ml loop.

5.4.2 Analytical silica TLC

It was found that the best eluent system for the crude TW extracts on silica TLC was chloroform containing 7-10% methanol. No spots could be seen under the UV lamp, but spots developed when sprayed with a solution of ceric ammonium acetate in dilute sulphuric acid followed by heating; the reference standard, triptolide, presented an apparently well resolved spot at an R_f of 0.8 while the reference standards tripdiolide and triptolide chlorohydrin, which had an R_f of 0.4-0.5, coeluted with many other components of the complicated TW405 mixture.

5.4.3 Preparative silica TLC

Based on the very good resolution of the ethyl acetate extract on high performance analytical silica plates, 170 mg of TW405 was plated on a 20 x 20 cm preparative silica plate and developed under the optimum conditions, 93:7 $CHCl_3$/MeOH. Unfortunately, the material streaked strongly on the preparative scale TLC plate using coarser particles: it presented a clear orange band (T2) at an R_f of 0.5 and a yellow band (T3) below it. The chromatogram was cut in 6 bands, T1 to T6, following the order of their increasing R_f values and 5 tests equivalents were sent to China.

Bioassay (Table 5-2) demonstrated that, at this higher dose level, material of intermediate polarity could be recovered from TW405 by preparative TLC and retained activity: animal tests proved T2 and T3 to be active. However, the quality of separation of the TLC bands was rather poor due to the complex nature of the mixture and low loading of the preparative TLC plates was found to be a limitation of this enrichment technique. After analysing the

6 TLC bands in comparison with the starting material TW405 on an HPLC ODS-column under the continuous gradient elution conditions described later in Chapter 5.4.4 (Figure 5-2), it was concluded that the active preparative TLC fractions were composed of very complicated multicomponent mixtures and that preparative HPLC would be necessary for efficient processing.

Table 5-2
The effects of 405 series on the fertility of male rats.+

Group (n)	Body wt gain (g)	Weight of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml	% w/w starting material	
Control(5)	121±25	1.70±0.20	90±5	86±10		
405(5)	91±26	1.70±0.10	2±4	28±15	100.0	A
405-T1(5)	109±43	1.60±0.09	85±5	71±5	30.76	
405-T2(5)	87±33	1.49±0.18	0	7±3	3.36	A
405-T3(5)	93±44	1.48±0.16	2±3	10±7	1.92	A
405-T4(5)	81±9	1.48±0.09	83±10	72±11	2.88	
405-T5(5)	76±30	1.61±0.12	82±3	74±5	2.33	
405-T6(5)	122±41	1.60±0.22	84±4	73±6	1.75	

+ Data expressed in x±SD, if applicable
A Active fraction

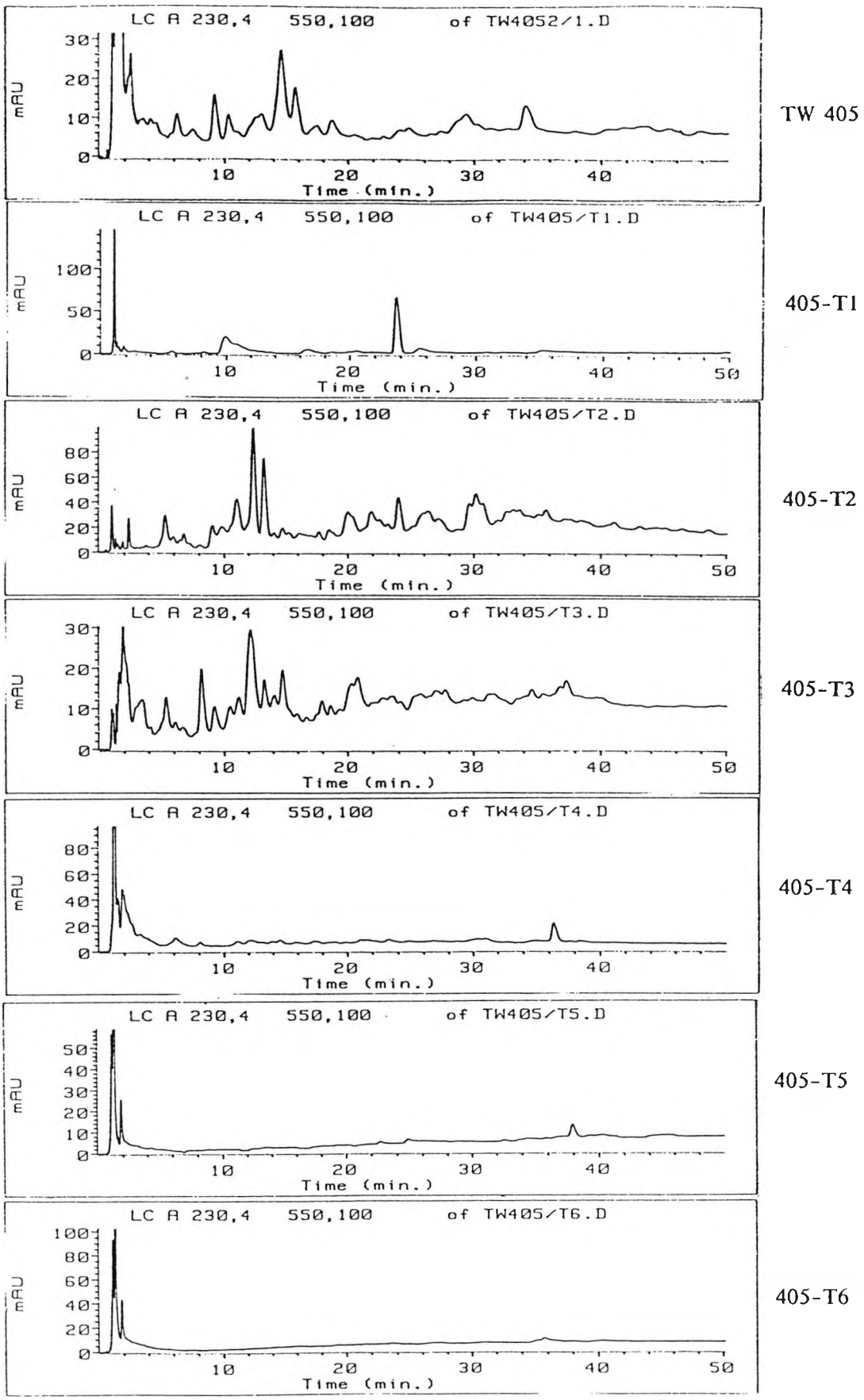


Figure 5-2. Comparison of the 6 preparative TLC fractions of TW405 (silica plate, 93:7 $CHCl_3$/MeOH) with the starting material 405. HPLC run on a 25 x 0.45 cm ODS-Hypersil 5 µm, eluted with a continuous gradient elution from 10/10/80 to 22/22/56 MeCN/MeOH/H_2O at 2 ml/min using a 25 ml mixing flask, monitored at 230 nm. Only 405-T2 and 405-T3 have male antifertility properties.

5. 4. 4 ODS Continuous gradient elution

A more extensive subdivision by HPLC had to be carried out. According to the antifertility tests in rats of the step gradient HPLC fractionation (Table 5-1), the activity in the ethyl acetate phase resided in compounds with an intermediate polarity. The resolution of this multicomponent mixture into single compounds would require a long running time under isocratic conditions. Step gradient achieved only limited separation of groups of components with similar polarity. To improve the separation across the region of intermediate polarity corresponding to the active subfraction, 415-2, a continuous gradient elution system was initially developed on the analytical scale and with an easy conversion to a HPLC preparative scale. An automatic gradient programmer, either low pressure or high pressure, was not available and we required a gradient generator which would be highly reproducible and which could be adapted for use on either the analytical (2 ml/min) or preparative (25-30 ml/min) scale with our pumps.

A cheap and simple low pressure gradient elution system was constructed by connecting a mixing chamber to an isocratic pump as shown in Figure 5-3.

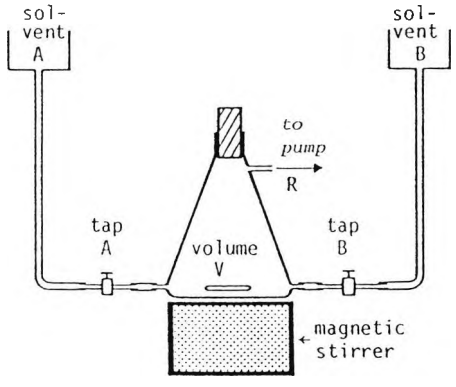


Fig. 5-3: Apparatus for generation of solvent gradients. [1]

The mixing chamber consists of a stoppered conical flask to which three side arms have been joined. Reservoirs of solvents A and B are connected to the two lower side arms via glass taps and PTFE tubing, and the mixing chamber is filled initially with solvent A. Elution is commenced with tap A open and tap B closed, the stirred contents of the flask being drawn directly into the pump. When it is desired to commence the gradient, tap A is closed and tap B is immediately opened. The concentration of the solvent B in the mixture then emerging from the mixing vessel increases with time (t) in an exponential manner, depending on the volume (V) of the mixing chamber and the flow rate (R_M) according to equation 5-1: [1]

$$\% \text{ B in mixture} = 100\ (1 - e^{-R_M t/V}) \quad \text{(Equation 5-1)}^{1}$$

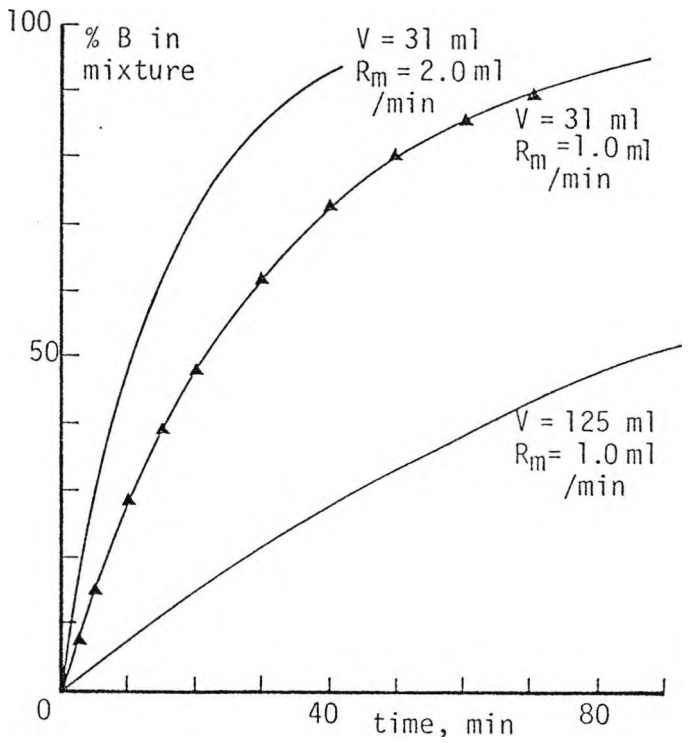


Figure 5-4. Gradient profiles produced by mixing vessels (calculated gradients). [1]

The profile of the gradient is changed by choosing mixing chambers of different volumes. Examples of gradients as calculated theoretically, can be seen in Figure 5-4. It has been reported that very good agreement is obtained between the calculated and the experimental gradient (within 1% at all points) when using a Waters twin piston pump.[1] Reproducibility was found to be excellent on both analytical and preparative scales.[1]

As the very polar and the least polar components of the ethyl acetate fraction had proved to be inactive (Table 5-1), the emphasis was directed in obtaining a reasonable separation of the medium polarity compounds. A gradient elution profile was studied using the same initial mobile phase of the previously described step gradient elution, 10/10/80 MeCN/MeOH/H_2O, and the mobile phase B being 50/50 MeCN/MeOH. The gradient profile was adjusted using different volume (10-100 ml) conical mixing flasks. Figure 5-5 presents an adequate and very reproducible separation using a 100 ml mixing flask on an analytical scale. 100% MeCN was used to wash the most polar components from the column at the end of each run.

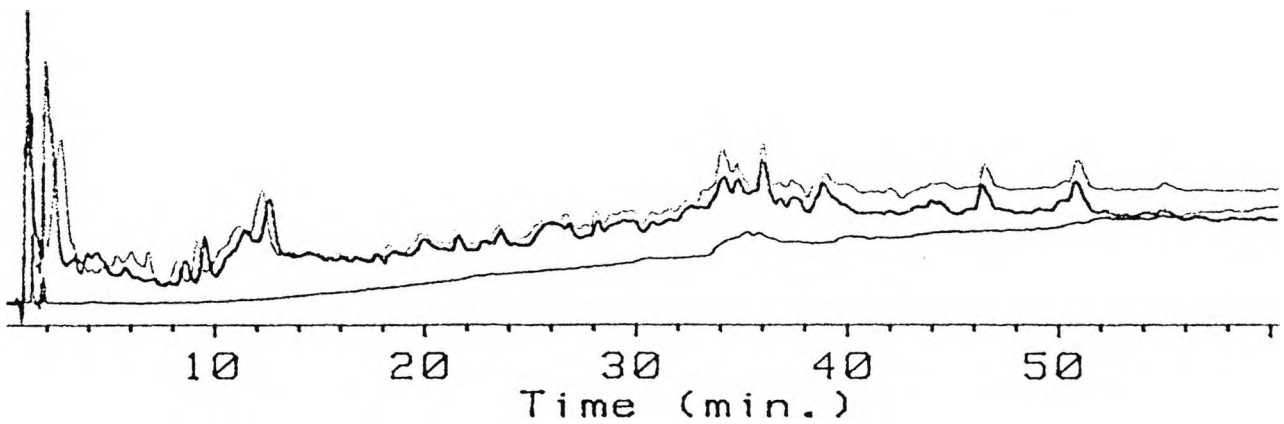


Figure 5-5. Reproducibility of continuous gradient elution profile of TW405 and comparison with a blank run, on a 25 x 0.45 cm Spherisorb-ODS1 5 µm, with a continuous gradient elution from 10:10:80 MeCN/MeOH/H_2O to 50:50 MeCN/MeOH using a 100 ml mixing flask at 2 ml/min and monitored at 230 nm.

A further improvement in resolution was obtained with a shorter gradient, 10/10/80 to 22/22/56 MeCN/MeOH/H_2O using a smaller mixing flask of 25 ml (Figure 5-6a).

The same mobile phase composition as in Figure 5-6a was applied to run a preparative separation using a 1" O.D. 30 cm long Hypersil-ODS 5 μm column with a 30 ml flow rate, which gave back-pressures up to 5500 psi. A 2 litre mixing flask was found to give an adequate separation as seen in Figure 5-6b. Using a 4 ml loading loop the loading capacity of the column was considered to be 140 mg of TW405 dissolved in methanol. 17 fractions were collected and coded 425-01 to 425-17, as shown in Figure 5-6b.

5 test equivalents, corresponding to 394 mg, of TW405 (ethyl acetate fraction) were processed. The subfractions were evaporated at temperature below 45°C to minimize any decomposition which might lead to loss of antifertility activity. After drying under vacuum, fractions were sent to China for animal tests along with a sample of crude TW405 as a positive control.

Antifertility activity was detected in fractions 425-01 and 425-08 (Table 5-3). Subfraction 425-08 was composed mainly of one single compound (Figure 5-8a) while the subfraction 425-01 was a very complicated mixture which needed improved separation.

Table 5-3
The effects of 425 series on the fertility of male rats. +

Group (n)	Body wt gain (g)	Weight of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml	% w/w starting material	
Control(5)	165±33	1.64±0.13	85±3	86±9		
405(5)	144±42	1.65±0.14	2±5	46±13	100.0	A
425-01(5)	142±35	1.58±0.33	5±4	28±3	30.76	A
425-02(5)	146±21	1.71±0.14	85±5	85±11	3.36	
425-03(5)	154±33	1.65±0.12	77±10	82±9	1.92	
425-04(5)	160±27	1.64±0.15	80±9	79±10	2.88	
425-05(5)	166±54	1.60±0.15	82±6	85±8	2.33	
425-06(5)	153±31	1.68±0.16	78±8	82±9	1.75	
Control(5)	159±29	1.61±0.14	88±5	78±19		
425-07(5)	148±28	1.55±0.07	82±4	69±11	1.61	
425-08(5)	155±27	1.50±0.19	7±3	27±14	2.95	A
425-09(5)	148±26	1.56±0.06	81±9	73±19	3.09	
425-10(5)	146±18	1.53±0.11	88±8	65±32	1.99	
425-11(5)	165±24	1.50±0.07	79±13	76±10	1.20	
425-12(5)	160±32	1.67±0.16	82±8	73±12	2.78	
425-13(5)	158±36	1.55±0.14	82±10	67±9	1.85	
425-14(5)	153±38	1.62±0.10	81±4	84±24	1.75	
425-15(5)	164±25	1.60±0.14	79±10	81±10	5.49	
425-16(5)	149±26	1.64±0.10	84±4	90±8	19.62	
425-17(5)	158±23	1.54±0.11	82±7	79±13	14.68	

+ Data expressed in x±SD, if applicable
A Active fraction

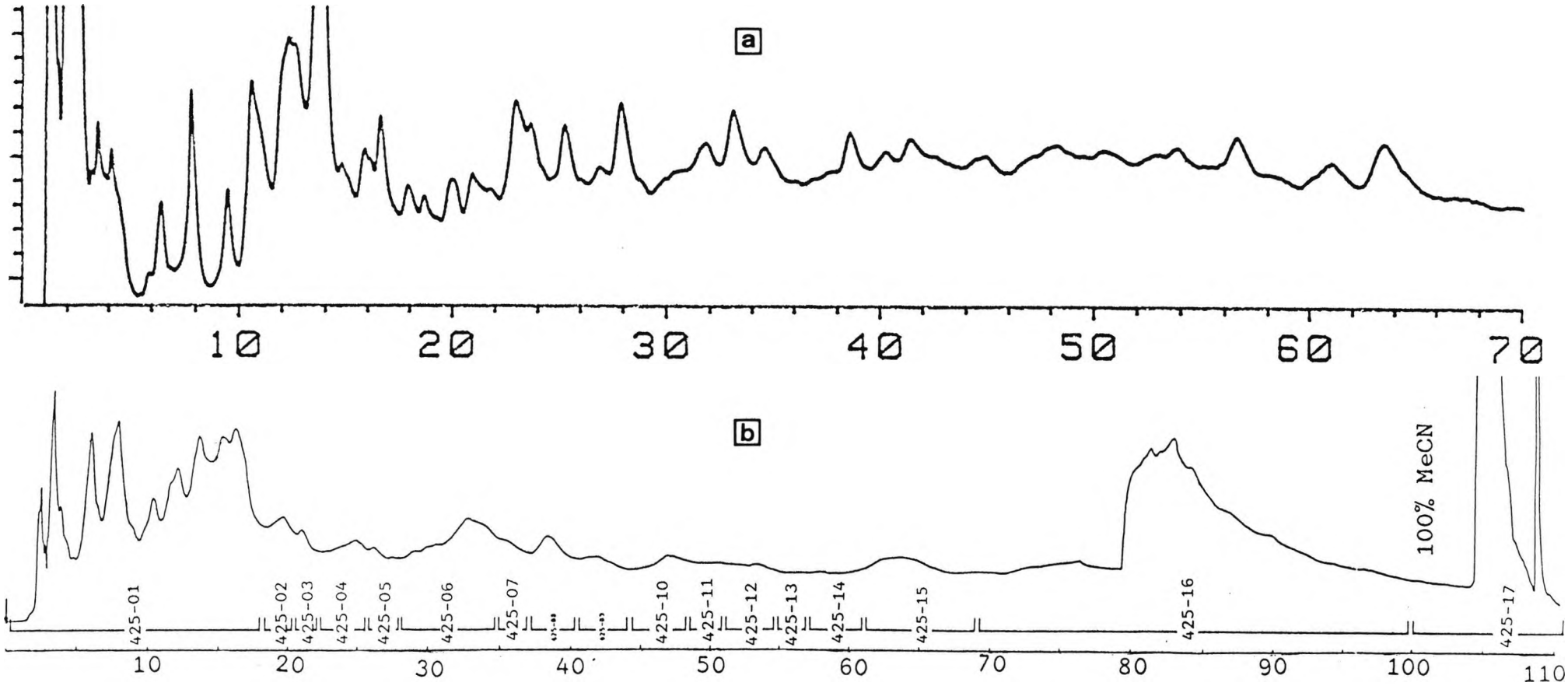


Figure 5-6. HPLC separation of 405 by continuous gradient elution on reverse phase (monitored at 230 nm). a) Analytical HPLC on 25 x 0.45 cm ODS-Hypersil 5 µm column, eluted by continuous gradient from 10:10:80 to 22:22:56 MeCN/MeOH/H_2O using a 25 ml mixing flask at 2 ml/min. b) Preparative HPLC separation into 17 fractions on a 1" O.D. x 33 cm ODS chemically bonded to silica-Hypersil 5 µm column, eluted by continuous gradient from 10:10:80 to 22:22:56 MeCN/MeOH/H_2O using a 2 l mixing flask at 30 ml/min, monitored at 0.02 AUFS using an analytical flowcell.

5.4.5 Selective separation of active subfractions 425-01 and 425-08

Five additional test equivalent of TW405 were chromatographed as in Section 5.4.4, but this time the collection was aimed at resolving the active fractions from the remaining non-active fractions, as seen in Figure 5-7; the first eluting fraction 435-01 was subdivided into 3 subfractions, coded 435-01-1, 435-01-2 and 435-01-3. Six subfractions in total were collected.

During the development of the optimum conditions to resolved the 435-01-3 fraction, problems were encountered and the fraction 435-01-1, 435-01-2 and 435-01-3 were lost. As not enough material from the TW tablet extract Series 400 remained, it was decided to continue the development of a separation method with the new material TW600 Series (See Chapter 5.6.3).

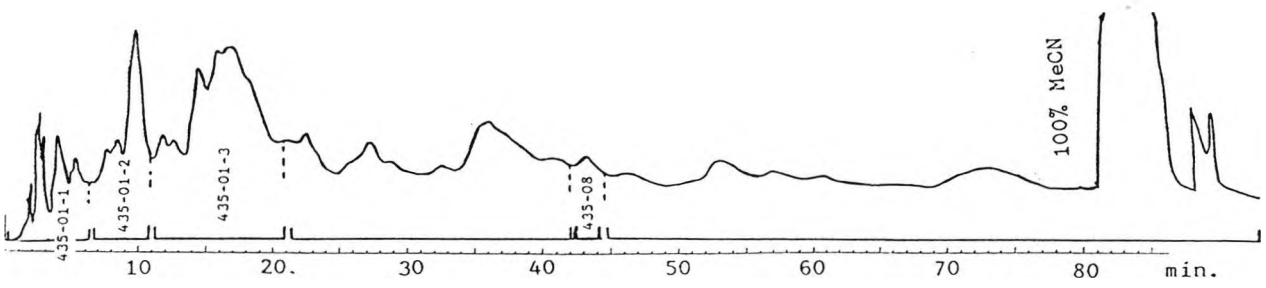


Figure 5-7. Collection of the active fractions 435-01 and 435-08 by selective fractionation of the active groups. 200mg sample was injected using a 4 ml loop on a 1" O.D. x 33 cm preparative ODS bonded on silica-Hypersil 5 µm column and eluted with continuous gradient elution from 10:10:80 to 22:22:56 MeCN/MeOH/H_2O at 30 ml/min using a 2 l mixing flask, monitored at 230 nm and 0.02 AUFS using an analytical flowcell. 435-01 is subfractionated in 435-01-1, 435-01-2 and 435-01-3.

5.4.6 Purification of active 435-08: development of an HPLC system.

The active fraction 425-08 eluted as one main component with partially resolved shoulders, as shown in Figure 5-8a. Under the same chromatographic conditions the starting material TW405 was also compared with the active "Compound X", isolated previously by V. Stacey (Fig. 5-8b). On standing on methanol, "Compound X" transformed slowly into an earlier eluting compound, when chromatographed on a ODS column using a MeOH/MeCN/H_2O mobile phase; both "Compound X" and its "transformation product" showed identical UV spectra (Fig. 5-9), indicating that the structure of both compound were very closely related (they contain the same chromophore).

An isocratic system was investigated on silica-ODS to avoid the reconditioning time needed for gradient elution chromatography. Optimum conditions for purification of the main component of this fraction were obtained using 28/72 MeCN/H_2O, which exposed this subfraction as a much more complicated mixture (Figure 5-10).

Subfraction 435-08 was purified on a semi-preparative scale following the isocratic conditions of Fig. 5-11 and the pure product 445-08 was analysed under identical isocratic conditions (Fig. 5-12, top left); the superimposition of the chromatographic peak of 445-08 and that of the transformation product of Compound X, combined to the perfect superimposition of their UV spectra (Fig. 5-12, bottom and top right, respectively), indicated 445-08 to be identical to the "transformation product" of Compound X. This finding had been previously observed during a comparison of the main component of 435-08 and "Compound X" (Fig. 5-8 & 5-10). Both compounds were shown to be very active in antifertility assays in China.

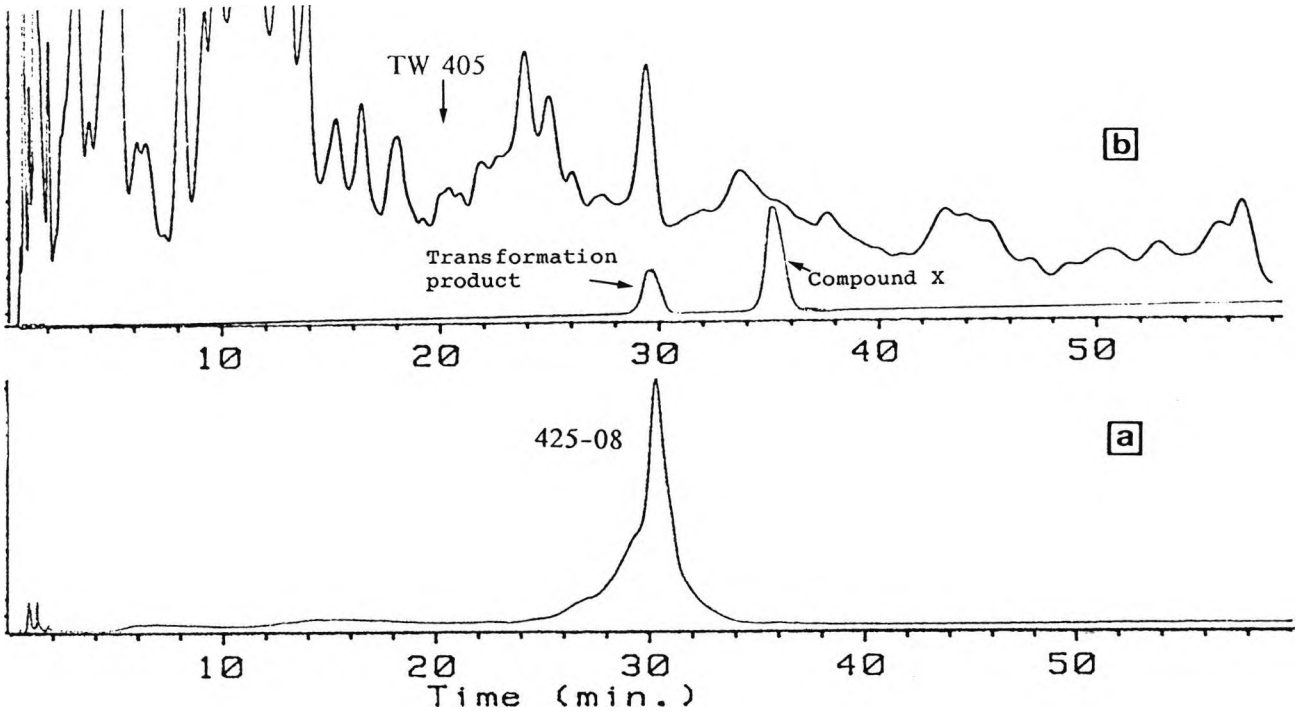


Figure 5-8. Purity assessment of the active fraction 425-08 (a) and comparison with the starting material 405 , and the active reference standard, "Compound X" (b), on a 25 x 0.45 cm Spherisorb-ODS1 5 µm column, eluted under continuous gradient from 10: 10: 80 to 22: 22: 56 MeOH/MeCN/H_2O using a 50 ml mixing flask at 2 ml/min, monitored at 230 nm.

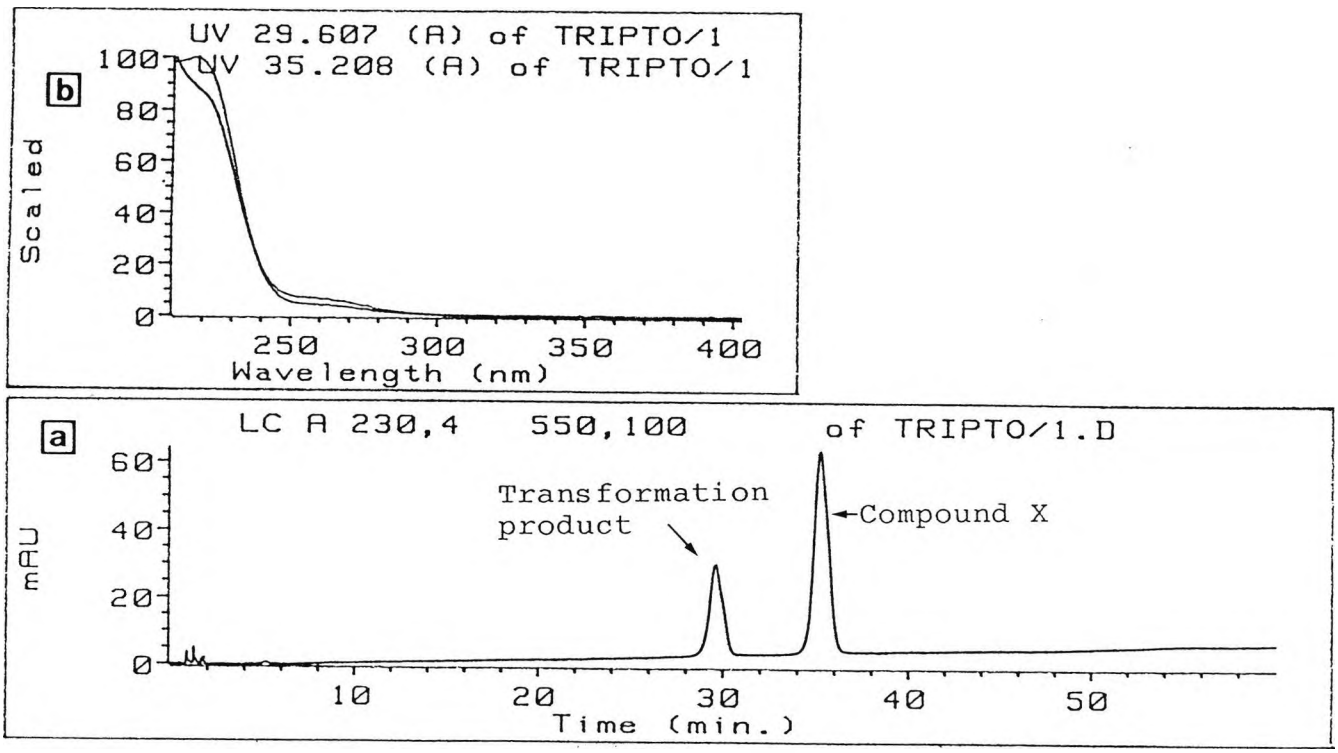


Figure 5-9. a) "Compound X", partially transformed on standing in methanol, chromatographed on a 25 x 0.45 cm Hypersil-ODS 5 µm HPLC column, eluted with a continuous gradient from 10: 10: 80 to 22: 22: 56 MeOH/MeCN/H_2O using a 50 ml mixing flask at 2 ml/min, monitored at 230 nm. b) UV spectra with the photodiode array detector of both, "Compound X" and its transformation product.

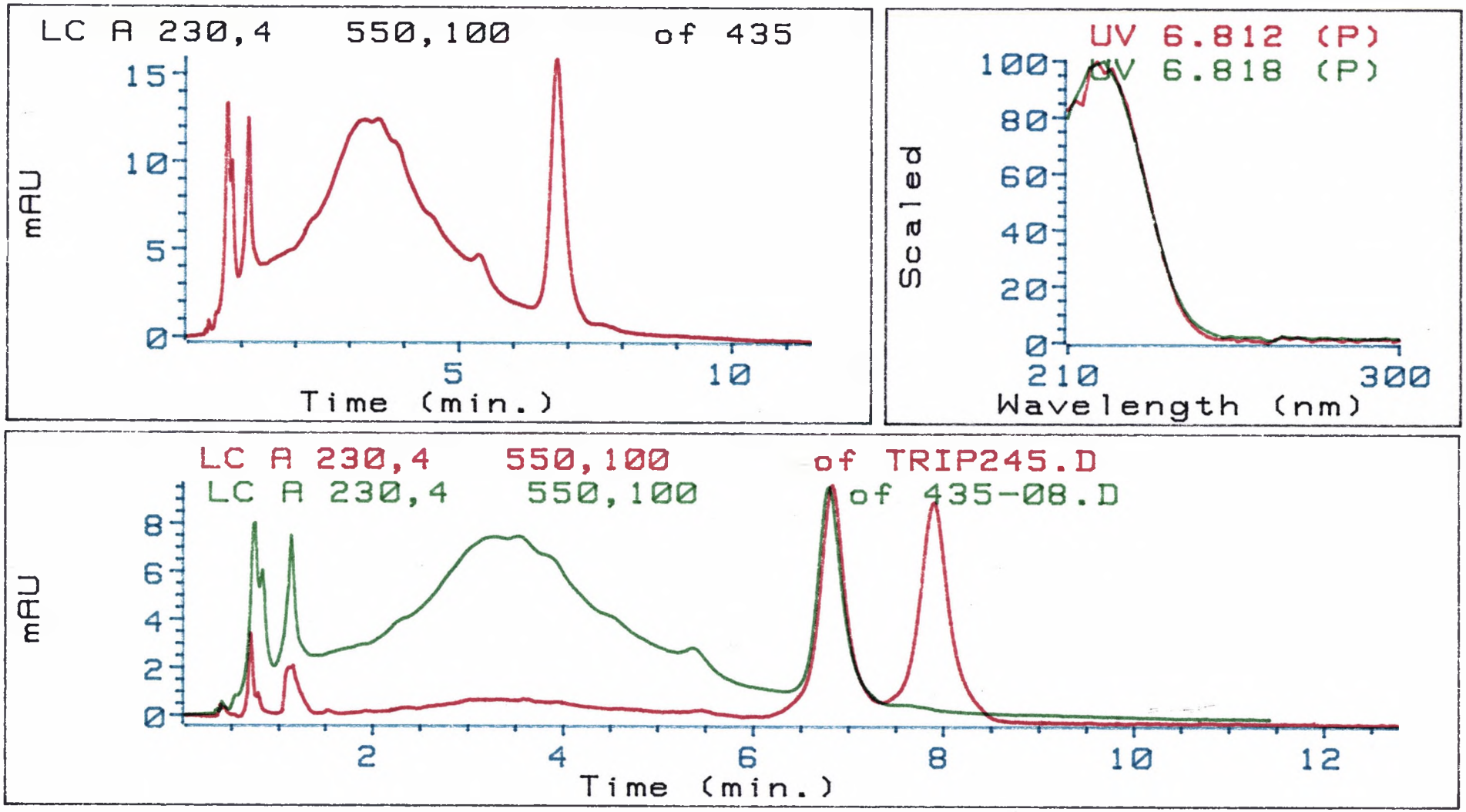


Figure 5-10. Isocratic HPLC separation method for 425-08, on a 25 x 0.45 cm analytical ODS-Hypersil 5 µm column, eluted with 28:72 MeCN/H_2O at 2 ml/min and monitored at 230 nm (Top left). (Top right): Superimposed UV spectra of the main component of 425-08 eluted at 6.8 min and that of the transformation product of "Compound X". (Bottom): Superimposed chromatograms of 425-08 and the partially transformed reference sample of "Compound X". The main component of 425-08 corresponds to the transformation product of "Compound X".

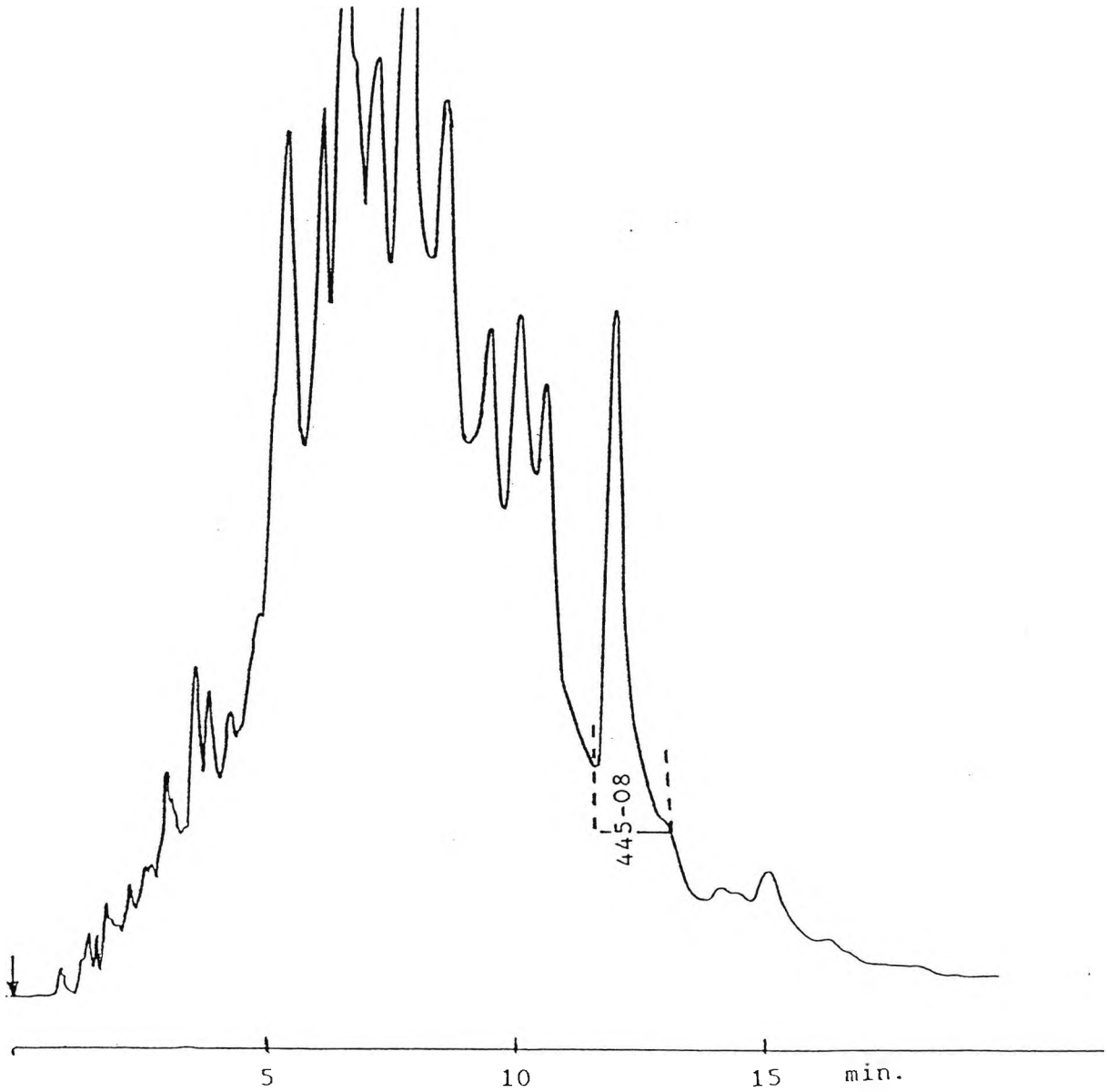


Figure 5-11. Semipreparative isocratic separation of the active fraction 425-08 on a 33 x 0.7 cm ODS-Hypersil 5 µm column eluted with 28:72 MeCN/H_2O at 5 ml/min and monitored at 230 nm at 0.02 AUFS to obtain subfraction 435-08. Loaded sample : 5 mg in MECN on a 100 µl injection loop.

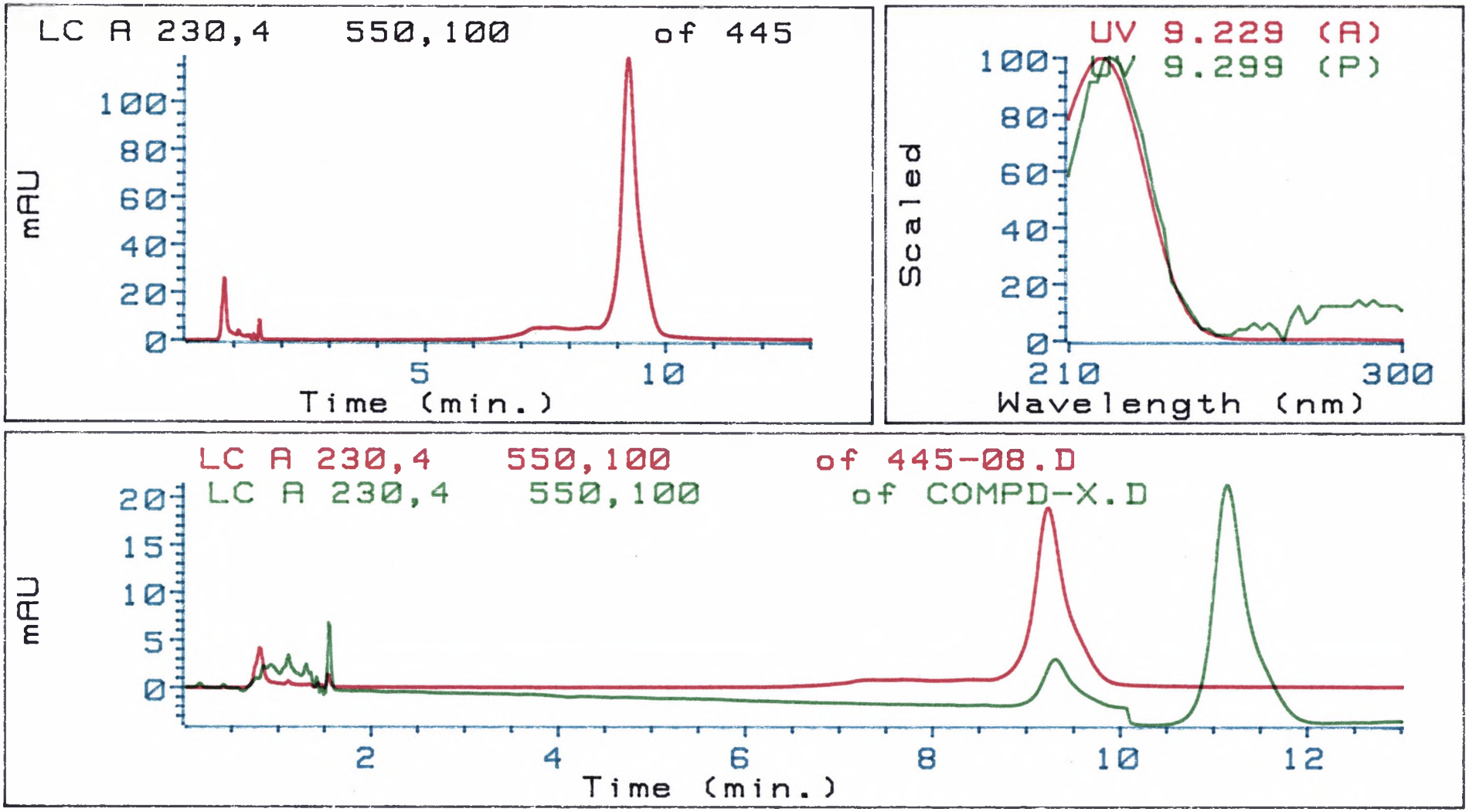
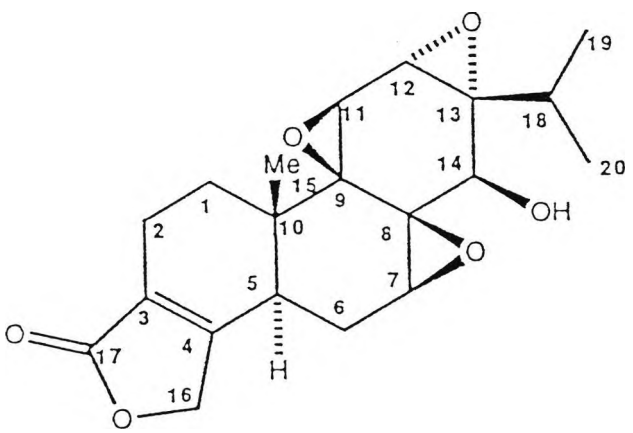


Figure 5-12. Purity assessment of 445-08, run under isocratic conditions with 25 x 0.45 cm Hypersil-ODS 5 µm column, eluted with 28:72 MeCN/H_2O at 2 ml/min, monitored at 230 nm (Top left). (Top right): Superimposed UV spectra of 445-08 and that of the transformation product of "Compound X". (Bottom): Superimposed chromatograms of 445-08 and the partially transformed reference sample of "Compound X". 445-08 corresponds to the transformation product of Compound X.

5.4.7 Structural elucidation of 445-08 and "Compound X"

Sample 445-08 and "Compound X" were sent to Dr. J.K.M. Sanders at Cambridge University for detailed spectroscopic examination. A high field ^1H NMR spectrum of sample 445-08 was compared with that for "Compound X". The NMR spectrum of sample 445-08 (Figure 5-13, Table 5-4) showed it to be fully consistent with the published data[2-4] for triptolide (1). The ^1H NMR spectrum of "Compound X" (Figure 5-14, Table 5-4) was also found to be similar to that of triptolide reported by Kutney et at,[4] however, there were large (>0.5 ppm) differences in some of the chemical shifts and "Compound X" had an additional singlet at δ 1.47; "Compound X" was found to differ from triptolide in the region of the 12,13-epoxide. Integration of the peak areas of the ^1H spectrum agreed with the number of protons calculated from the mass spectrum, i.e. 25. "Compound X" was revealed by mass spectrometry to be a chlorohydrin of triptolide. It is suspected that this is probably a product of the work-up conditions employed in obtaining the plant extract. Interestingly, on standing triptolide chlorohydrin in the $CDCl_3$ NMR solution (over a period of weeks), triptolide is formed in a clean reaction.



(1)

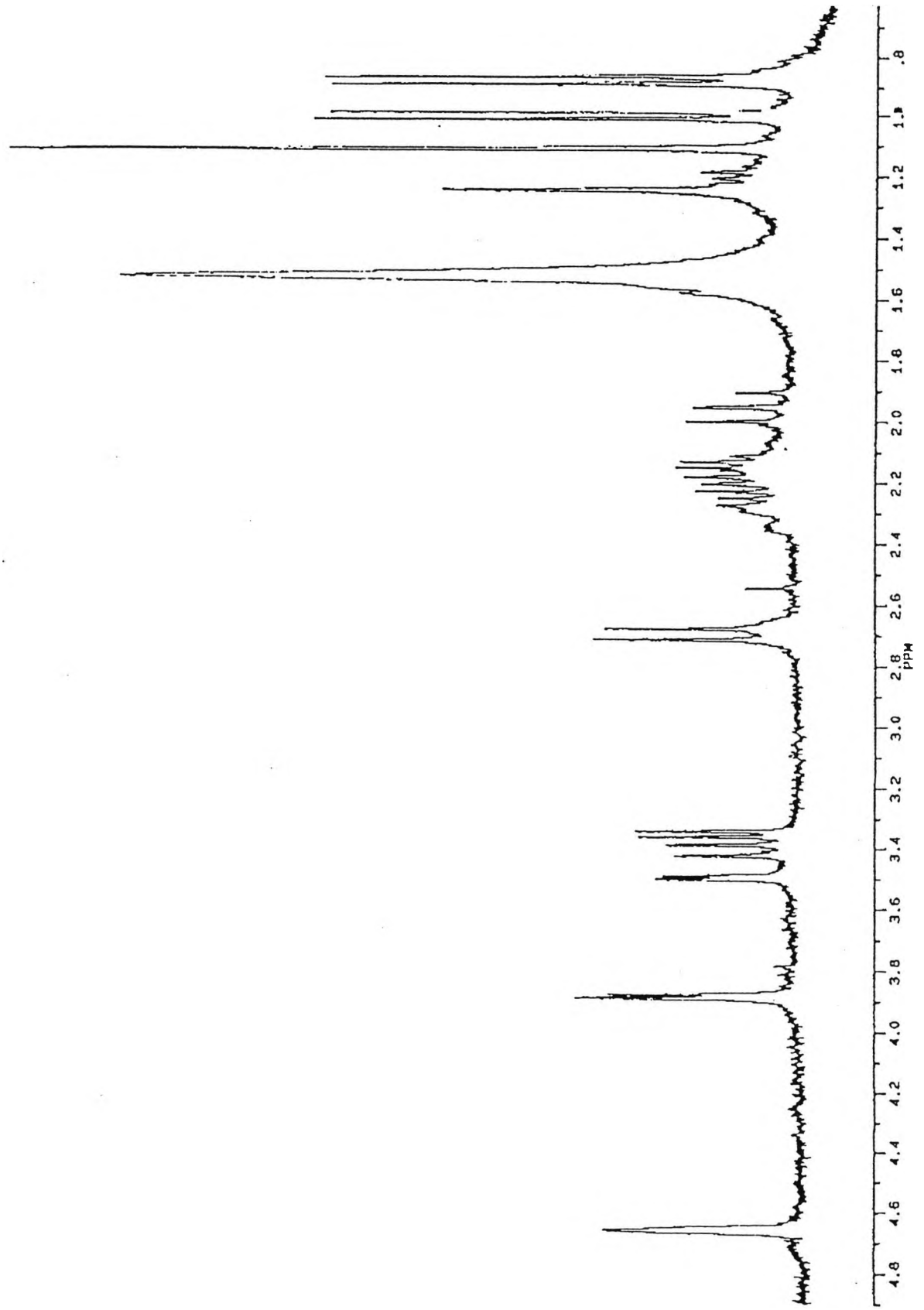


Figure 5-13: ^{1}H NMR of triptolide (445-08)

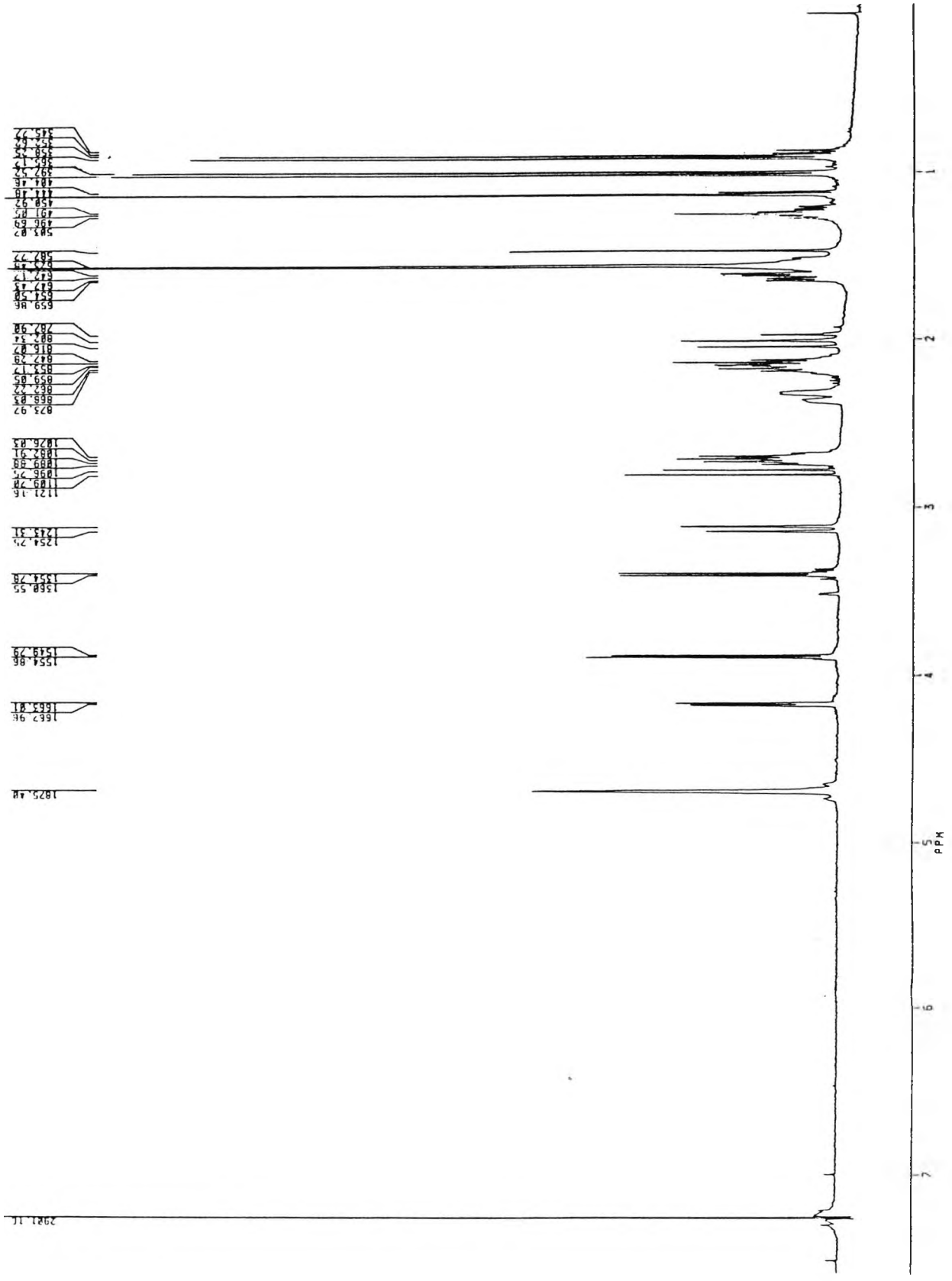


Figure 5-14: ^{1}H NMR of triptolide chlorohydrin (Compound X)

Table 5-4
1H chemical shifts and couplings for triptolide and its 12, 13-chlorohydrin in CDCl3

	TRIPTOLIDE		12,13-CHLOROHYDRIN		
Proton	Chemical Shift (ppm)	Multiplicity & coupling (Hz)	Chemical Shift (ppm)	Multiplicity & coupling (Hz)	homonuclear correlation
1α	1.21	o	1.24	ddd 12, 12 & 5	1β, 2β, 2α
1β**	1.55	o	1.63	dd 12 & 5	2β, 1α
2α	2.31	bd 16.4	2.33	dd 16.8 & 5	2β, 1α
2β**	2.14	o	2.14	m incl. 16.8 & 5	2α, 1α
5	2.68	o	2.71	m	6α, 6b
6α	2.14	dt 14.8, 5.6, 5.6	2.14	dt 14.9, 5.9 & 5.9	5, 6β, 7
6β**	1.95	dd 14.8, 13.0	2.00	dd 14.9, 13.0	5, 6β
7	3.35	d 5.6	3.39	d 5.9	6α
11	3.87	d 3.4	3.88	d 7.3	12
12	3.48	dd 3.4, 0.8	4.16	dd 7.3, 1.4	11, 14
13(OH)			1.47	s	
14	3.41	dd 11.1, 0.8	3.12	dd 12.5, 1.4	12, 14-OH
14-OH	2.68	d 11.1	2.78	d 12.5	14
15	1.12	s	1.13	s	
16(α,β)	4.65	bs	4.69	bs	2α
18	2.23	sep 6.9	2.71	m	16, 17
19***	1.01	d 6.9	0.90	d 7.0	15
20***	0.87	d 6.9	1.00	d 7.0	15

s, singlet; d, doublet; t, triplet; sep, septet;
m, multiplet; o, obscured
** assigned as axials by NOE difference experiments
*** methyl groups.

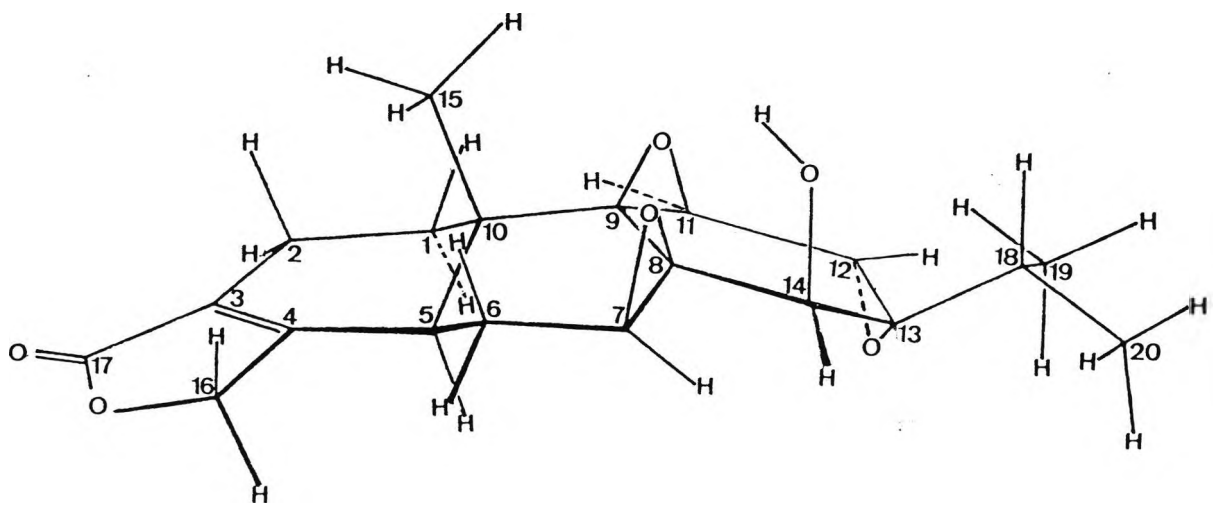
The NMR work reported here covers, ^1H-^1H COSY and nOe difference experiments obtained at Cambridge by Dr. J. K. M. Sanders' student, C. Pearce, on the sample of triptolide (TW445-08) and used to establish its identity and preferred conformation. Comparisons were made with the COSY and nOe results[5] obtained at Cambridge by another student, S. Amor, on the "Compound X" isolated by V. Stacey. The mass spectrometry, described below, covers the electron impact (EI) and chemical ionization (CI) experiments performed on TW445-08 and "Compound X", which led to the identification of the latter.

The ^1H NMR spectra of TW445-08 and "Compound X", Fig. 5-13 and Fig. 5-14 respectively, are very similar (Table 5-4); both were assigned by a standard combination of ^1H-^1H COSY and nOe difference experiments. Scalar and dipolar coupling connectivities revealed by these two experiments on triptolide are summarised in Table 5-5. It should be mentioned that only a small amount of TW445-08 was available for nmr (1 mg), presumably allowing only the stronger nOes to be observed.

Table 5-5
H connectivities established by 1H-1H COSY and nOe difference experiments with triptolide in CDCl3

Proton	Proton connected by scalar coupling	Proton connected by nOe enhancement
1	1′	1′
1′	1, 2, 15	1, 11
2	1′, 16	2′
2′		
5	6′	
6	6′, 7	7
7	6	
11	12	12, 1′
12	11, 14	11
14	12, 14-OH	
14-OH	14	Saturation transfer to water
15	1′	1, 6′
16	2	
18	19, 20	
19	18	12, 18
20	18	14, 18

From the COSY spectrum of triptolide (Table 5-5) the ^1H connectivities allow the signals to be gathered together in four separate groups: 1, 1′, 2, 2′, 15, 16; 5, 6, 6′, 7; 11, 12, 14, 14-OH and 18, 19, 20; consistent with the proposed structure of triptolide. The ^1H-^1H coupling constants and nOe difference results suggest the preferred conformation (2), assigning 1, 2 and 6 as equatorial protons and 1′, 2′ and 6′ as axials. Taking into account the known stereochemistry of triptolide, protons 5 and 7 conform to being axial and equatorial respectively, and proton 11, 12 and 14 to being in the distorted orientation expected from having three epoxide groups in such close proximity. From the reported work[3] the 14-OH is thought to make a hydrogen bond with the 9, 11-epoxide. This may be consistent with the observed saturation transfer to water from the 14-OH during the nOe experiment (Table 5-5), the 14-OH being in slow exchange although water was found to be present in the sample.



(2)

Mass spectrometry was used to obtain further data on both TW445-08 and "Compound X". Aliquots from the NMR solutions of samples 445-08 and "Compound X" were examined first in EI and the in CI mode. TW445-08 under EI conditions

showed the highest significant ion to be at m/z 360, corresponding to the expected triptolide molecular ion. The first fragmentation, the loss of OH, gave an ion at m/z 343. From here there was an ion at virtually every mass, increasing in intensity, down to the limit of the scan at m/z 50.

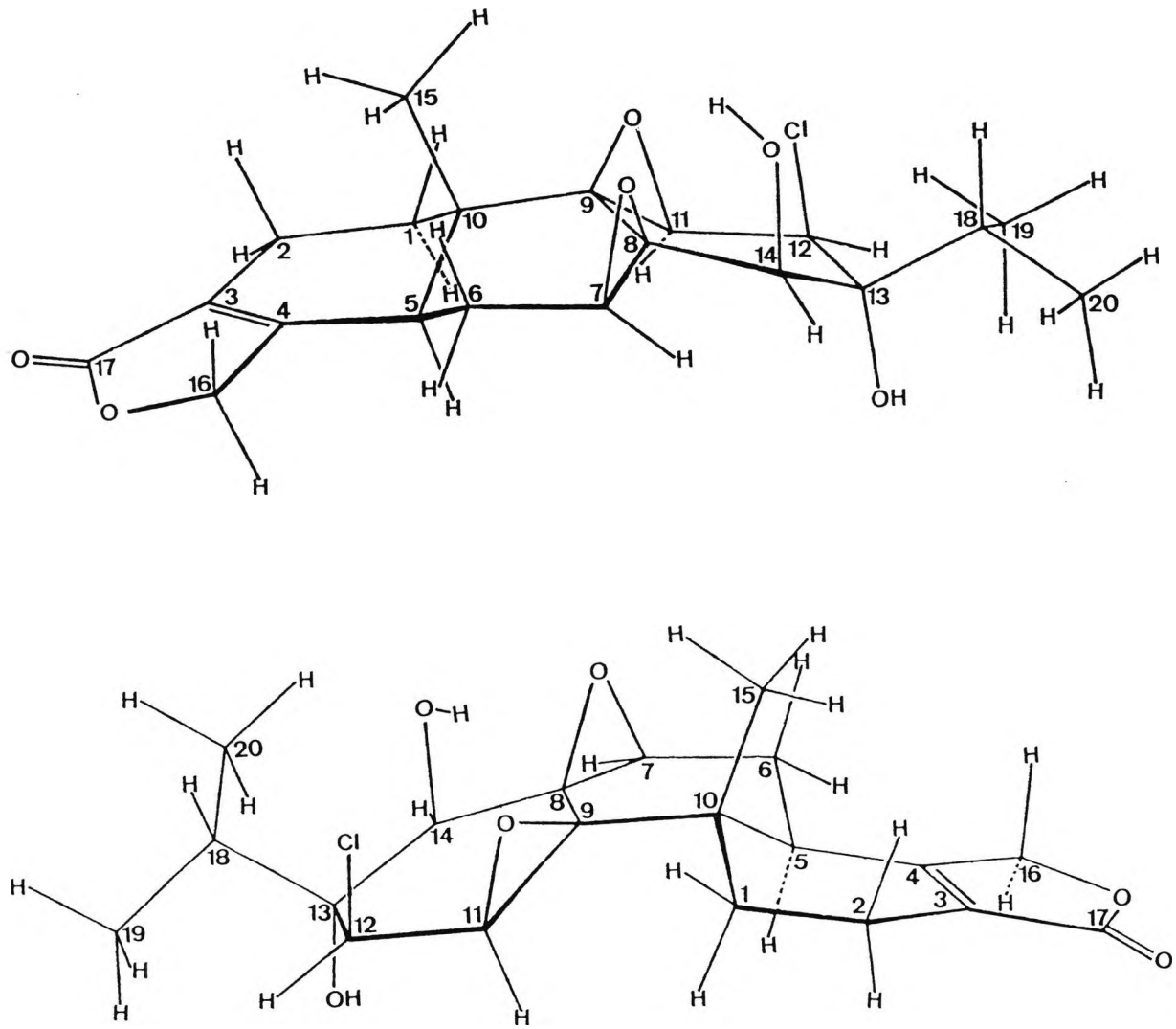
When "Compound X" was examined, the highest significant ions occurred at m/z 396 and 398, revealing a typical mono-chloro isomer pattern (intensities ca. 3:1). Compared to the base peak, which appeared at m/e 55, the m/z 396 ion was about 20% relative intensity. The next highest significant ion occurred at m/z 361 (non-chlorinated) which would obviously correspond to the loss of Cl from m/z 396/398. From here the spectrum closely resembles that of triptolide with the ions tending to increase in intensity with decreasing mass. Clearly, the ions at m/z 396 and 398 could correspond to the molecular ion of a chlorohydrin derivative and CI was used to examine the compound further. First, to establish good CI conditions for the proposed chlorohydrin, triptolide was examined and showed a strong pseudo-molecular ion at m/e 361. Using the same conditions, the derivative was then tried and indeed pseudo-molecular ions at m/e 397/399 (mono-chloro) resulted.

The structure of "Compound X" was initially studied by double quantum filtered (DQF) COSY (Table 5-4). Assuming the carbon skeleton was the same as triptolide, it was possible to make tentative assignments based on chemical shifts, coupling constants and coupling patterns for the proton resonances of rings A, B, the α, β-unsaturated lactone and the isopropyl group as follows. The signal at δ 4.69 of a pair of tightly coupled protons is typical of the CH_2 group at carbon 16. The 3H singlet at δ1.13 is typical of Me_{15}. The two upfield 3H

doublets at δ 0.90 and δ 1.00 coupled to a single proton at δ 2.71 are due to the methyls and central proton of the isopropyl group respectively. Only partial assignment of the remaining protons in ring C was possible. There are two pairs of coupled proton doublets. The 1H doublets coupled to each other at δ 3.88 and δ 4.16 are probably due to H_{11} and H_{12}, whilst the 1H doublets at δ2.78 and 3.12, are probably due to a geminal H and OH coupled to each other such as those at the carbon 14. This left just the singlet at δ 1.47 to be assigned. A complete list of assignments from the proton spectrum and homonuclear correlations obtained from DFQ COSY is given in Table 5-4. The ^{1}H NMR spectrum of "Compound X" (Fig. 5-14, Table 5-4) shows that the only significant chemical shift differences occur in the region of the 12,13-epoxide; signals from 12, 14, 14-OH and 18 all shifting to greater or lesser extent. The coupling between 11 and 12 increases from 3.4 Hz in triptolide to 7.3 Hz in triptolide chlorohydrin. These observation are consistent with the opening of the 12, 13-epoxide.

Taking into account the NMR evidence it was clear that the derivative was the 12,13-chlorohydrin of triptolide. A close inspection of the DQF COSY reveals a five-bond 1.4 Hz coupling between H_{12} and H_{14}. Thus, in the absence of any π-systems, the σ-orbitals of the CH protons must overlap. This W coupling is a common feature of proton spectra of sugars and shows that the conformation of ring C is a slightly distorted envelope, as shown in (3). No other stereochemistry or conformation allows the CH σ-orbitals of H_{12} and H_{14} to overlap or explains the nOe's observed so well. H_{12}, H_{14} and the isopropyl group are predominantly in the equatorial conformation by virtue of the enhancements seen from H_{12} to

H_{11}, Me_{19} to H_{12} and Me_{20} to H_{14}, This then implies that the chlorine and the hydroxyl substituents are axial whichever way round they lie on C_{12} and C_{13}. Chemical sense suggests the epoxide is ruptured by nucleophilic attack of the chlorine via a SN2 mechanism. On steric grounds it would seem more probable that the attack takes place at C_{12}, leading to the chlorine being axial and also in turn the hydroxyl on C_{13}. Thus, this independent chemical argument suggests the same relative stereochemistry described in the tentative nOe-based proposal.



(3)

Front and back view of a three dimensional computer drawing of triptolide chlorohydrin, produced by graphic plot using "desk top molecular modeller", Oxford University Press, Version 1.2.

Definitive proof of this preferred assignment of triptolide chlorohydrin, as illustrated in (3) was finally supplied by nOe difference experiments (Table 5-6). The most indicative data is the saturation transfer from the doublet at 2.78 to the water signal and the singlet at δ 1.47, which shows that this doublet is hydroxyl proton at the carbon 14 and that the singlet is also a hydroxyl group. The doublet at δ 3.12 is therefore H_{14}. This is confirmed by a 9% nOe from Me_{20} to H_{14} and a 7% nOe from H_{14} to H_7 (Table 5-6). The sharp singlet hydroxyl is in slow exchange with water and must be exchanging with a rate slower that 28 Hz to be so sharp (this being the difference in chemical shift between the two resonances). Thus coupling to another proton would be expected to be observed or, if the coupling was small, to broaden the signal. Thus, this hydroxyl is evidently one that is attached to a quaternary carbon. If this is so, nOe's from both isopropyl methyl groups (Me_{19} and Me_{20}) and H_{12} show it is attached to C_{13} (hindered rotation of the isopropyl group causes the diastereotopic methyls to give nOe's to different sides of the molecule. Thus an nOe from both rules out the possibility that the OH is attached to C_{12} with a very small coupling to H_{12}).

Table 5-6 : nOe data from nOe difference experiment on Triptolide chlorohydrin

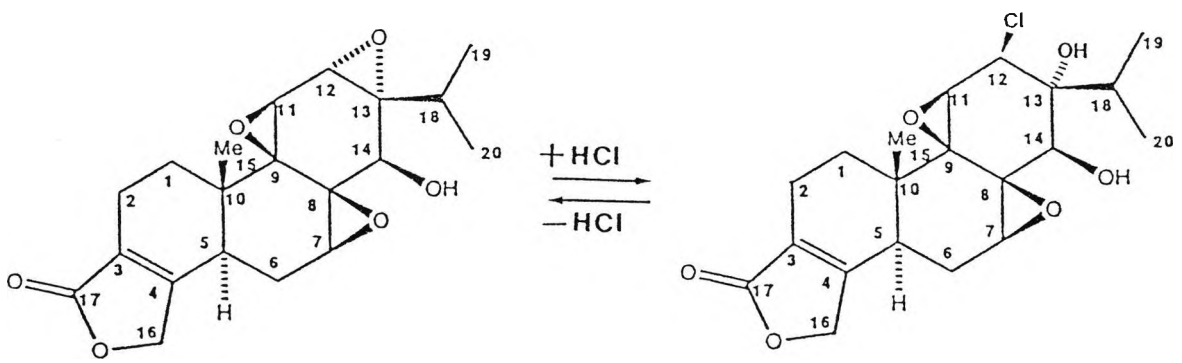
Resonance/s Saturated	nOe to proton	%	nOe to proton	%
1α	1β	9	2α	3
	5 & 18	1	11	8
1β, H_2O & 13(OH)	11	5	14(OH) sat trans	
2β & 6α	2α	12	5 & 18	3
	7	7	15	1
5 & 18	2β & 6o	9	19	1
			20	1
6β	2β & 6α	5	7	1
	20	1		
7	2b & 6α	5	14	1
11	12	5	1α	4
			1β	3
12	13(OH)	2	19	3
14	7	7	20	2
14(OH)	H_2O sat trans		13(OH) sat trans	
15	1β	3	2β & 6α	3
	6β	3		
19	12	7	13(OH)	5
	5 & 18	10		
20	14	9	13(OH)	3
	5 & 18	9		

5.4.8.1 Transformation of triptolide to triptolide chlorohydrin

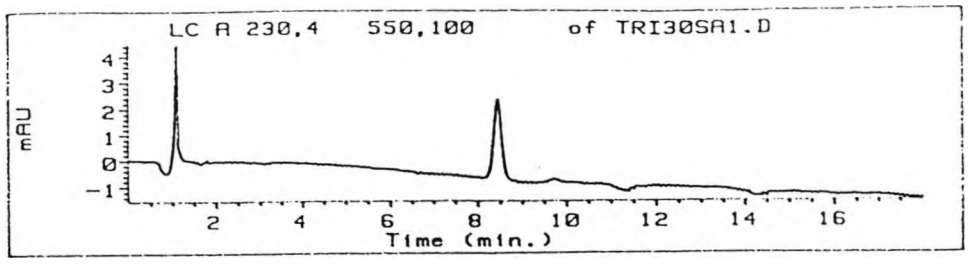
It is believed that triptolide chlorohydrin is formed during the extraction of material from the roots of Tripterygium wilfordii or its subsequent processing for tablet manufacture. An experiment was carried on to form triptolide chlorohydrin by reacting triptolide with HCl (Scheme 5-1).

During reaction of triptolide with dry HCl gas dissolved in MeCN, aliquots were taken at intervals and evaporated to dryness and redissolved in MeCN prior to HPLC injection. In Figure 5-15, triptolide can be seen to be transformed gradually to triptolide chlorohydrin ("Compound X"), this transformation being detectable after 3 minutes and progressing with increasing time. Therefore it is likely that triptolide chlorohydrin is a by-product of the initial extraction procedure.

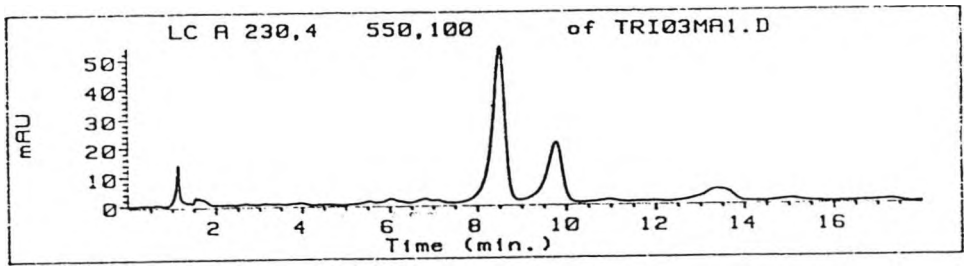
As noted earlier, transformation in the opposite direction was also observed, samples of triptolide chlorohydrin in solutions of various solvents (MeOH, NMR solvents) tending to transform on standing to gradually regenerate triptolide.



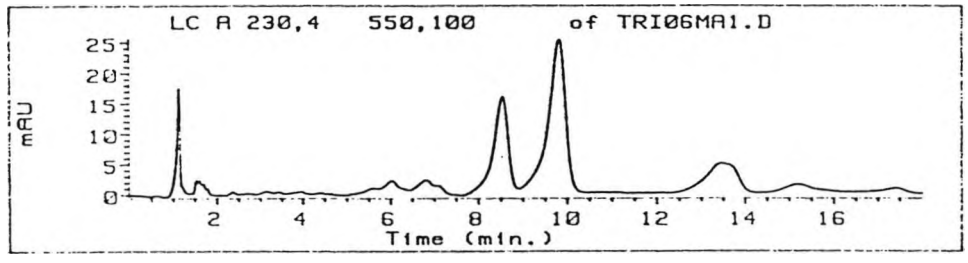
Scheme 5-1



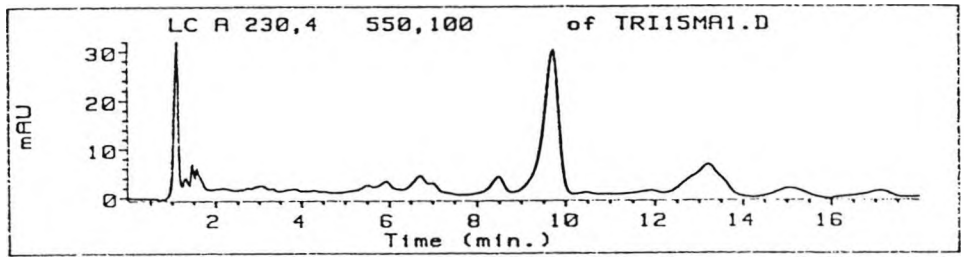
30 seconds



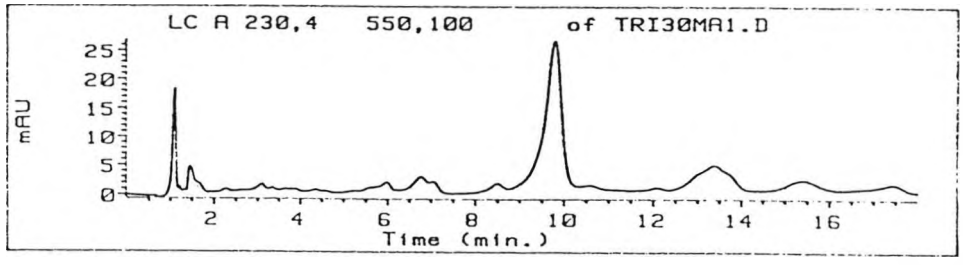
3 minutes



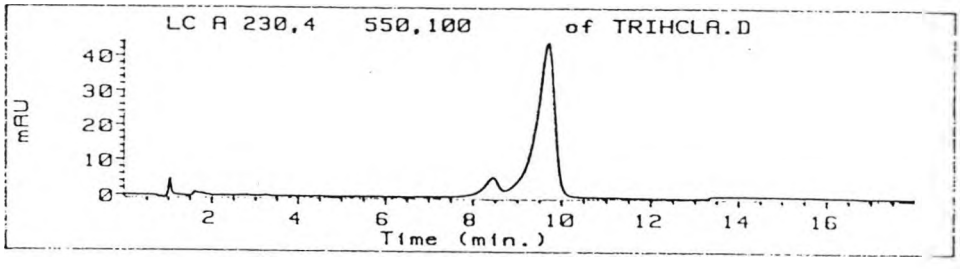
6 minutes



15 minutes



30 minutes



Triptolide chlorohydrin partially transformed to Triptolide

Figure 5-15. Transformation of triptolide to triptolide chlorohydrin by reaction with dry HCl gas in MeCN at 30 seconds, 3, 6, 15 and 30 minutes. Comparison with "Compound X" identified as triptolide chlorohydrin which has been partially transformed to triptolide on standing on MeOH. Chromatographed on a 25 x 0.45 cm Hypersil-ODS 5 µm column, eluted with 28:72 MeCN:H_2O at 2 ml/min, monitored at 230 nm.

5.4.9 Further subfractionation of 435-01-3: development of an HPLC system

The active subfraction 425-01, is a very complex multicomponent mixture of the most polar material of the ethyl acetate fraction and it accounts for 30% (Table 5-3) of this fraction. As the step gradient results confirmed (Table 5-1), the activity should be only located in the least polar subfraction 435-01-3.

There are various factors to consider in the method development, one being to improve the selectivity of the separation by either changing the solvents used in the mobile phase or by using a different stationary phase. Another factor to consider, is to simplify the removal of the solvent under mild conditions; evaporating highly aqueous fractions under mild conditions requires a high vacuum and it is a very slow process. The use of highly aqueous fraction on a silica based material is not recommended, if it can be avoided, as some silica can dissolve in water.

An interesting observation is that the first fraction, 415-1, of the multistep gradient separation of TW405 showed no antifertility properties (see Table 5-1), while the first fraction of the continuous gradient elution 425-01, showed clear antifertility activity (see Table 5-3). Both fractions were eluted on the same stationary phase and with the same initial mobile phase mixture, 10:10:80 MECN/MeOH/H_2O, but while 415-1 was eluted isocratically, the mobile phase strength to elute 425-01 was continuously increased. Therefore, the active fraction could logically not be the first eluting polar compounds, such as 425-01-1 and 425-01-2, but probably would be the least polar compounds of the multicomponents mixture, 425-01-3. HPLC analysis of the 3 step

gradient fractions and the starting material TW405 run under the continuous gradient elution condition can be seen in Figure 5-16. It was therefore decided to concentrate all the efforts to further separate the probable active subfraction 435-01-3, while the other two subfractions would be sent for antifertility tests with no further work done to them.

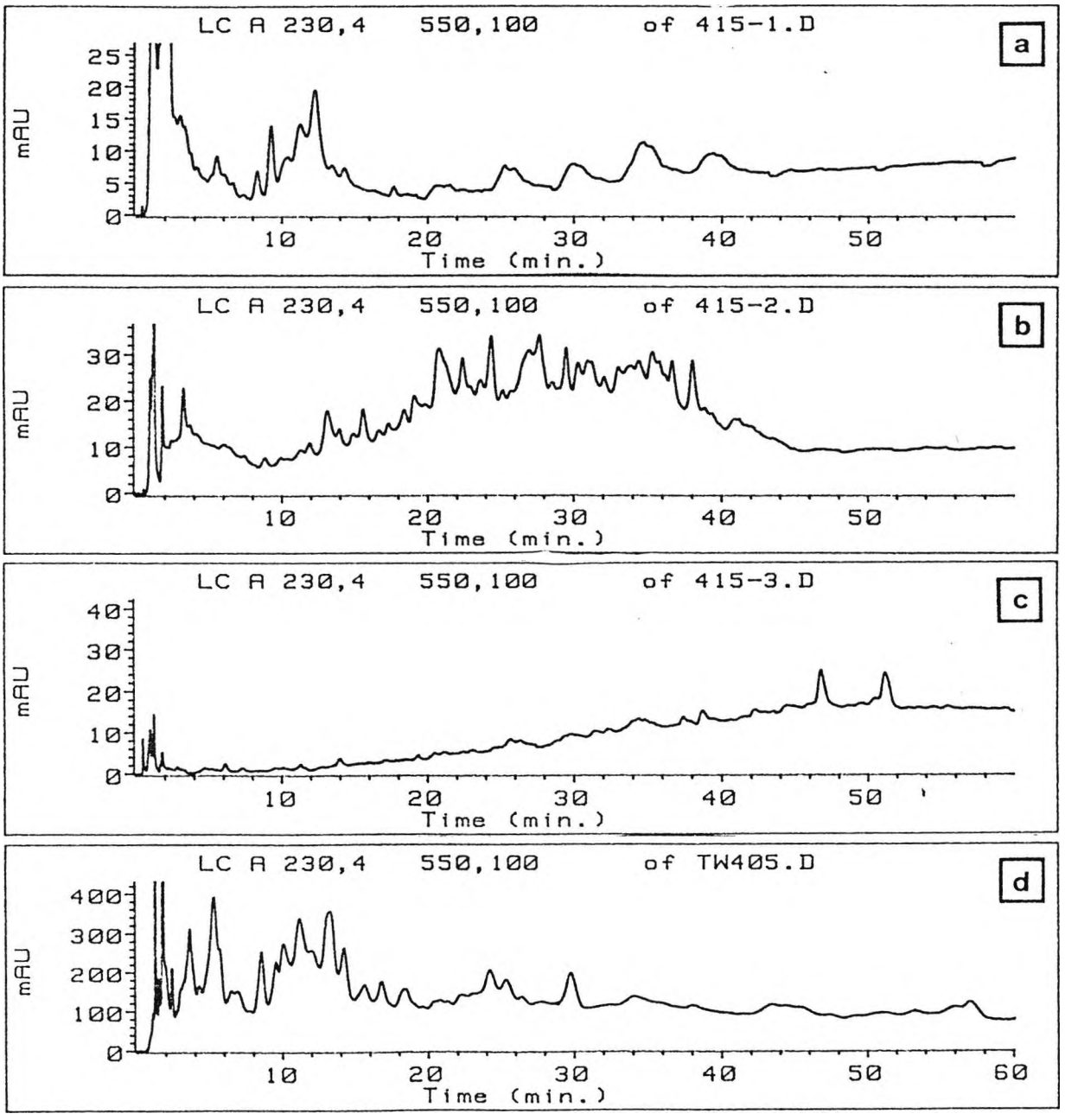


Figure 5-16. Fractions 415-1 (a), 415-2 (b), 415-3 (c) and starting material TW405 (d) run under continuous gradient elution on a 25 x 0.45 cm ODS-Hypersil 5 µm column from 10:10:80 to 22:22:56 MeCN/MeOH/H_2O using a 50 ml mixing flask at 2 ml/min, monitored at 230 nm. 415-2 is the only active fraction.

CN-silica : normal phase

Alternative normal phase chromatographic conditions were investigated with a bonded polar phase. Bonded polar phases offer the advantages of a less polar stationary phase than silica although they can be also used in the normal phase mode, which would be preferred for the preparative fractionation. Aminopropyl-silica used under normal phase conditions, had proved unacceptable due to low recovery (see Chapter 4.5.7.1). The selectivity of a cyanopropyl-silica stationary phase was investigated with mobile phases containing solvents with low UV cut off points, such as hexane and dichloromethane and with the help of modifiers such as methanol, acetonitrile, isopropanol or tetrahydrofuran. Dichloromethane tended to produce a noisy baseline, as the transmission at the required 230 nm is relatively low. Figure 5-17 shows the optimum resolution found using this stationary phase. This separation is totally unacceptable and the use of the cyanopropyl silica stationary phase was abandoned.

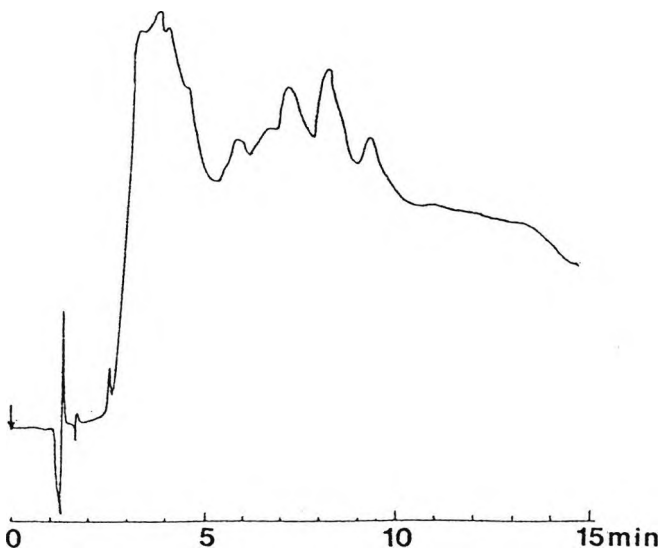


Figure 5-17. Normal phase HPLC separation of subfraction 435-01-3 in a 25 x 0.45 cm Ultrasphere-cyano 5 µm column, eluted with 85:5:15 Hexane/CH_2Cl_2/MeOH at 2 ml/min, monitored at 230 nm.

Nitro-silica : normal phase

Nitro-Nucleosil is believed to be a para-nitrophenyl propylsilane bonded to a Nucleosil silica. The selectivity of this material used under normal phase conditions, showed very good resolution of the subfraction 435-01-3. The presence of methanol seems to be necessary to obtain a good separation and it was observed that THF had a better effect than isopropanol on the resolution (Figure 5-18). Despite the improved resolution and the advantage of the use of normal phase solvent, the packing of a preparative scale column would be unaffordable if we needed to buy the commercially bonded nitro silica stationary phase.

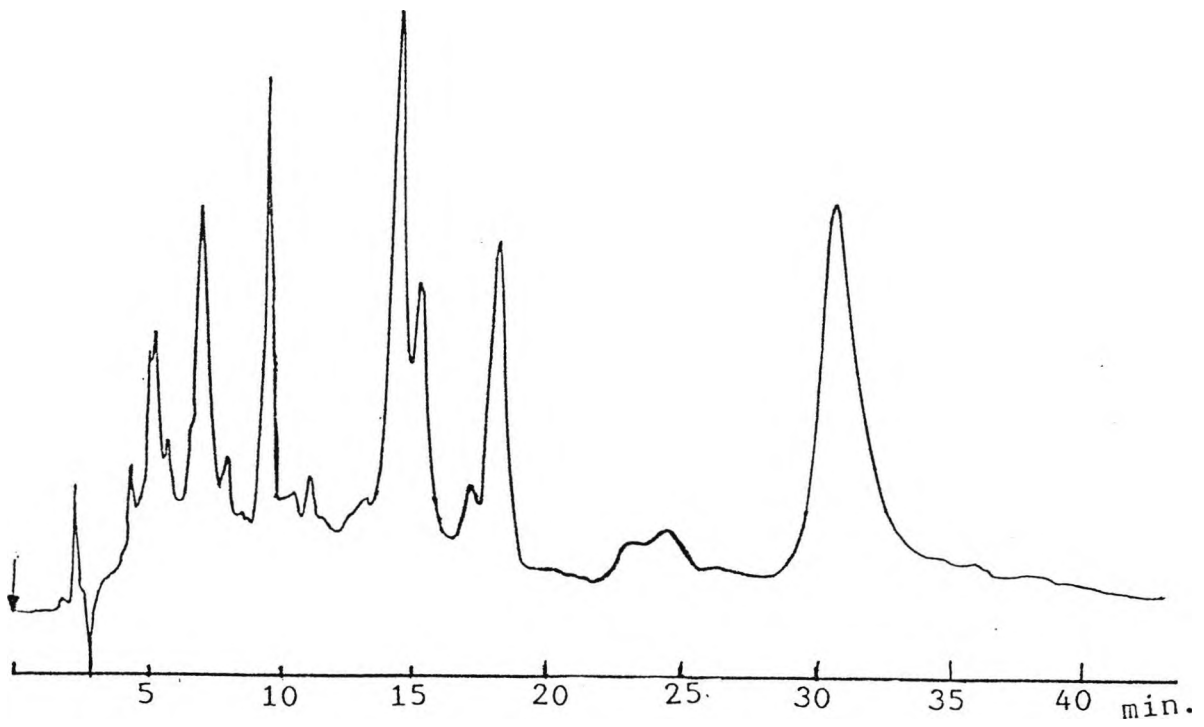


Figure 5-18. Normal phase HPLC separation of subfraction 435-01-3 in 25 x 0.45 cm Nucleosil-nitro bonded 5 µm column, eluted with 80:5:5:10 Hexane/CH_2Cl_2/THF/MeOH at 2 ml/min, monitored at 230 nm.

It was decided to investigate the more general application of this material in other more complex extracts, the extracts used being those of Series 600 (See page 271)

ODS silica: reverse phase

To simplify the separation of the least polar compounds (Fraction 435-01-3) from the active Fraction 425-01, an isocratic method on silica ODS was investigated on an analytical scale. The selection of a mobile phase is traditionally a process of trial and error, finding a single solvent or solvent mixture that provides appropriates k' values for the solutes to be separated.

Another approach to easily and rapidly design the optimum eluent is the use of the selectivity triangle. This method is based on the use of the eluotropic series, which allocates a solvent polarity index to each solvent.

Solvent	Polarity (P)
Acetonitrile	6.2
Methanol	6.6
Tetrahydrofuran	4.2
Water	9.0

It should be noted that the highest P value represents the weakest solvent for reverse phase. For solvent mixtures the composite eluent strength is represented by P', which is calculated as follows:

$$P' = F_aP_a + F_bP_b + F_cP_c$$

where F_a = the volume fraction of pure solvent a

P_a = The solvent strength of solvent a

Therefore an eluent of a given strength can be obtained with several different solvent combinations. Thus, when one eluent system fails to resolve all components of a sample, other solvents may be selected and the appropriate proportions calculated quickly and easily.

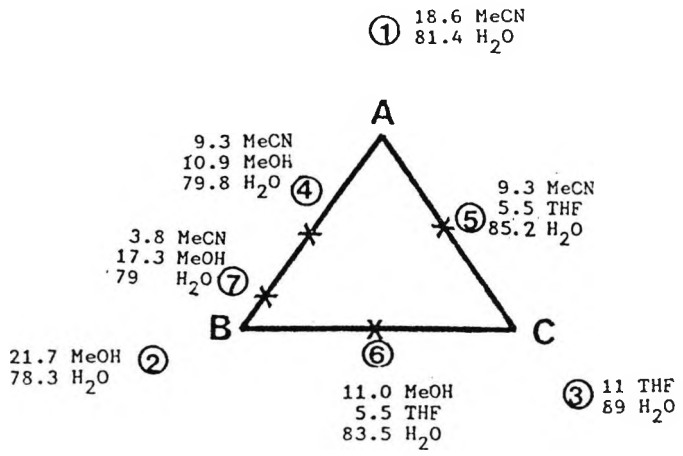


Figure 5-19. Selectivity triangle for reverse phase chromatography, using solvent mixtures containing MeOH, MeCN and THF, all with an eluent strength (P′) of 8.48.

The polarity was calculated for the initial 10/10/80 MeCN/MeOH/H_2O solvent combination used to elute the subfraction 435-01-3, as follows :

$$P' = 0.8x9.0 + 0.1x6.6 + 0.1x6.2 = 8.48$$

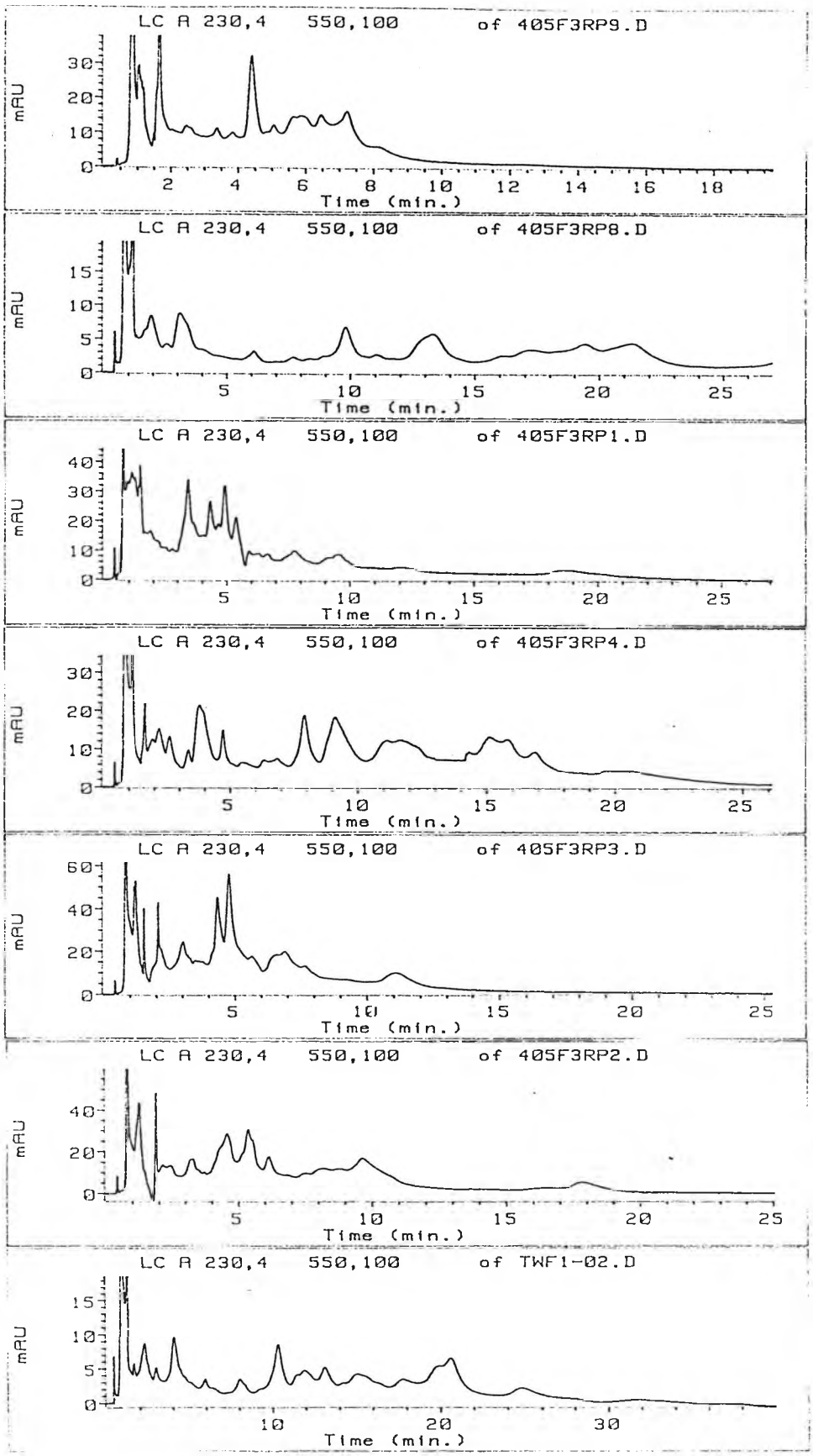
Maintaining this polarity, the 3 apex compositions were calculated:

A (MeOH/H_2O) P′ = 8.48 = 6.6x + **9**(1-x) 21.7MeOH/78.3H_2O

B (MeCN/H_2O) P′ = 8.48 = 6.2x + **9**(1-x) 18.6MeCN/81.4H_2O

C (THF/H_2O) P′ = 8.48 = 4.2x + 9(1-x) 10.8THF/89.2H_2O

Once the three apex composition have been calculated, the others solvent composition can be simply read from the graphic triangle (Fig. 5-19) since all values vary linearly. Any point from the graph (Fig. 5-19) has an eluent strength value P′ of 8.48, and hence all seven chromatograms run with these calculated eluent mixtures will yield k′ values in the same region for this separation (Fig. 5-20). The best resolution is obtained with the solvent mixture 4 of the triangle (10.9/9.3/79.8 MeOH/MeCN/H_2O).



1 18.6 MeCN
81.4 H_2O

2 21.7 MeOH
78.3 H_2O

3 11 THF
89 H_2O

4 9.3 MeCN
10.9 MeOH
79.8 H_2O

5 9.3 MeCN
5.5 THF
85.2 H_2O

6 11.0 MeOH
5.5 THF
83.5 H_2O

7 3.8 MeCN
17.3 MeOH
79 H_2O

Figure 5-20. HPLC chromatograms of the subfraction 435-01-3 eluted with the mobile phase compositions corresponding to the 7 points chosen of the selectivity triangle (Figure 5-19) on a 25 x 0.45 cm Spherisorb-ODS2 5 μm column, monitored at 230 nm.

An improved resolution of subfraction 435-01-3 (Figure 5-22) was obtained with the mobile phase calculated at the central point of a new selectivity triangle with a higher P′ value of 8.88 (Fig. 5-21).

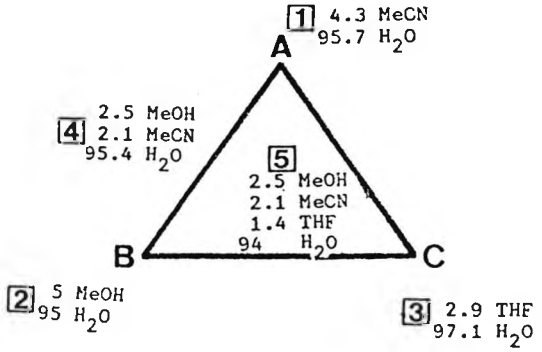


Figure 5-21. Selectivity triangle for reverse phase chromatography, using solvent mixtures containing MeOH, MeCN, and THF, all with an eluent strength (P′) of 8.88.

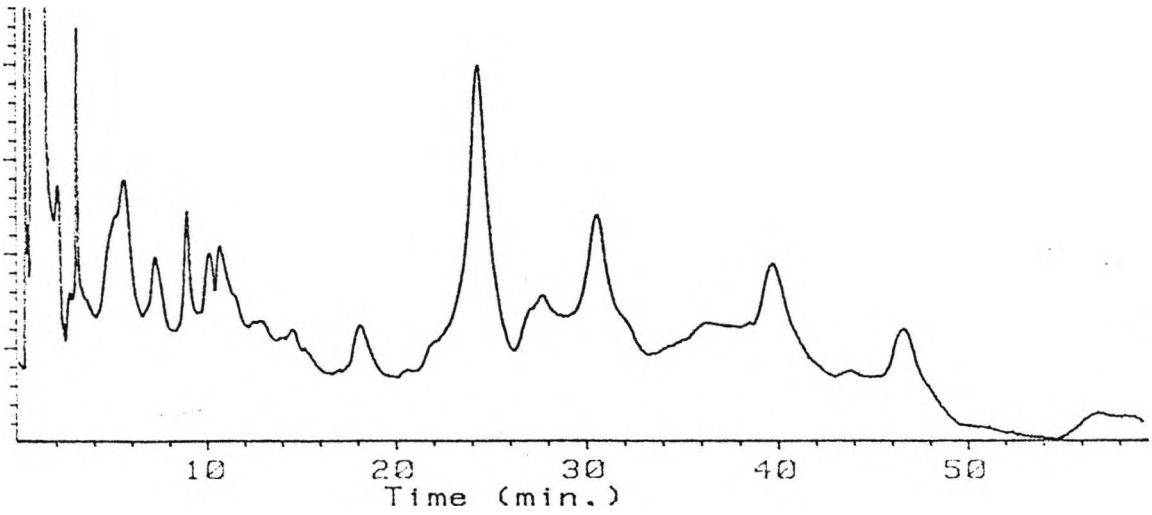


Figure 5-22. HPLC chromatogram corresponding to point 5 of the selectivity triangle in Figure 5-21, to achieve the optimum separation for 435-01-3 on a 25 x 0.45 cm ODS2-Spherisorb 5 µm column, monitored at 230 nm. Mobile phase 2.5:2.1:1.4:94 MeOH/MeCN/THF/H_2O, with a P′ of 8.88.

5.5 Fractionation of the aqueous soluble fraction

The aqueous soluble fraction TW402 proved to have a high antifertility activity (Table 4-3 to 4-5). A similar development protocol was followed to that which had been used to subfractionate the ethyl acetate fraction in Chapter 5.4. It was found that this fraction was not very soluble in cold water, and that the material could only dissolve in water after prolonged warming and sonication. This material was largely insoluble in any organic solvent.

5.5.1 ODS Step gradient

On an ODS-packing material, no isocratic system could be found to separate satisfactorily the many components of the aqueous fraction. Hence, a two step gradient was developed similar to that used to fractionate TW405 but with a more polar mobile phase (Figure 5-23a). Reproducibility of the chromatogram was also remarkably good.

The analytical conditions were easily transferred to a preparative scale (1" O.D. column) by increasing the flow rate to around 30 ml/min and injecting in a 4 ml loading loop around 750 mg of TW402 dissolved in water by warming. Three subfractions, coded 422-1, 422-2 and 422-3, were collected as indicated in Figure 5-23b and evaporated to dryness at temperatures below 40°C under reduced pressure.

Five test equivalents of the aqueous phase TW402 were processed and the three subfractions were sent to China for animal testing. The recovery of the subfractionation was 91% of the original material.

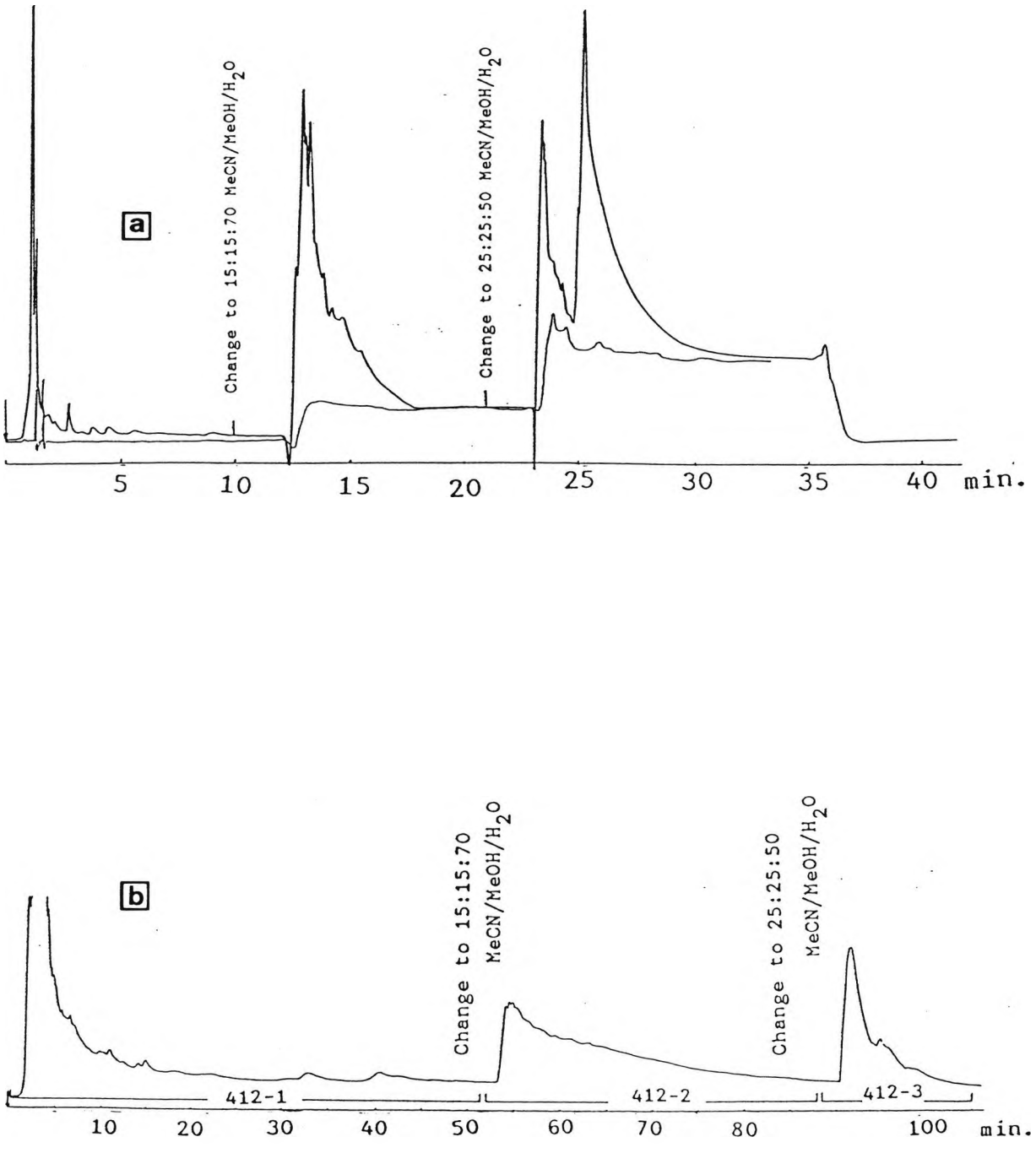


Figure 5-23. Multistep gradient separation of TW402 on ODS. a) Analytical HPLC on a 25 x 0.45 cm ODS-Hypersil 5 µm column, eluted with 7:7:86 MeCN/MeOH/H_2O for 0-10 min., 15:15:70 MeCN/MeOH/H_2O for 10-22 min., and 25:25:50 MeCN/MeOH/H_2O for 22-35 min, at 2 ml/min, monitored at 230 nm. b) Preparative separation on a 1" O.D. x 33 cm ODS bonded to silica-Hypersil 5 µm column, eluted with 7:7:86 MeCN/MeOH/H_2O for 0-50 min., 15:15:70 MeCN/MeOH/H_2O for 50-85 min., 30:30:40 MeCN/MeOH/H_2O for 85-105 min., at 30 ml/min monitored at 230 nm. Loading: 750 mg in MeCN on a 4 ml injection loop.

The last and least polar fraction, accounting for only 0.4% of the fractionated material (Table 5-7), was demonstrated to be inactive; the remaining two fractions were active as an antifertility agent in rats. There was a suspected overlap of eluted compounds between the first 422-1 and second 422-2 fraction, as a consequence of using the step gradient chromatographic technique. Therefore, there could be more than one active compound, or alternatively only one active compound with a polarity value such that it would elute in both fractions.

Table 5-7
The effects of 412 series on the fertility of male rats.+

Group(n)	Body wt gain (g)	Weight of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml	% w/w starting material	
Control(5)	138±41	1.62±0.06	84±4	95±18		
402(5)	146±34	1.70±0.17	4.0±4.2	18±8	100.0	A
412-1(5)	98±38	1.26±0.37	0.1±0.2	16±15	98.25	A
412-2(5)	127±31	1.62±0.15	3.3±4.2	24±14	1.34	A
412-3(5)	146±28	1.58±0.11	87±3	94±8	0.41	

+ Data expressed in x±SD, if applicable
A Active fraction

5.5.2 ODS Continuous gradient

A continuous gradient solvent elution system on an analytical scale was developed to avoid overlapping of fractions and to improve considerably the separation of the two more polar fractions. Because many compounds were so polar, it was found necessary to start the gradient elution with 100% H_2O and increase the solvent strength to 12/12/76 MeCN/MeOH/H_2O over one hour, ensuring the elution of the remaining compounds with 30/30/60 MeCN/MeOH/H_2O. A 50 ml conical mixing flask was found to achieve an adequate gradient profile (Figure 5-24a). Reproducibility of the chromatograms was found to be very good.

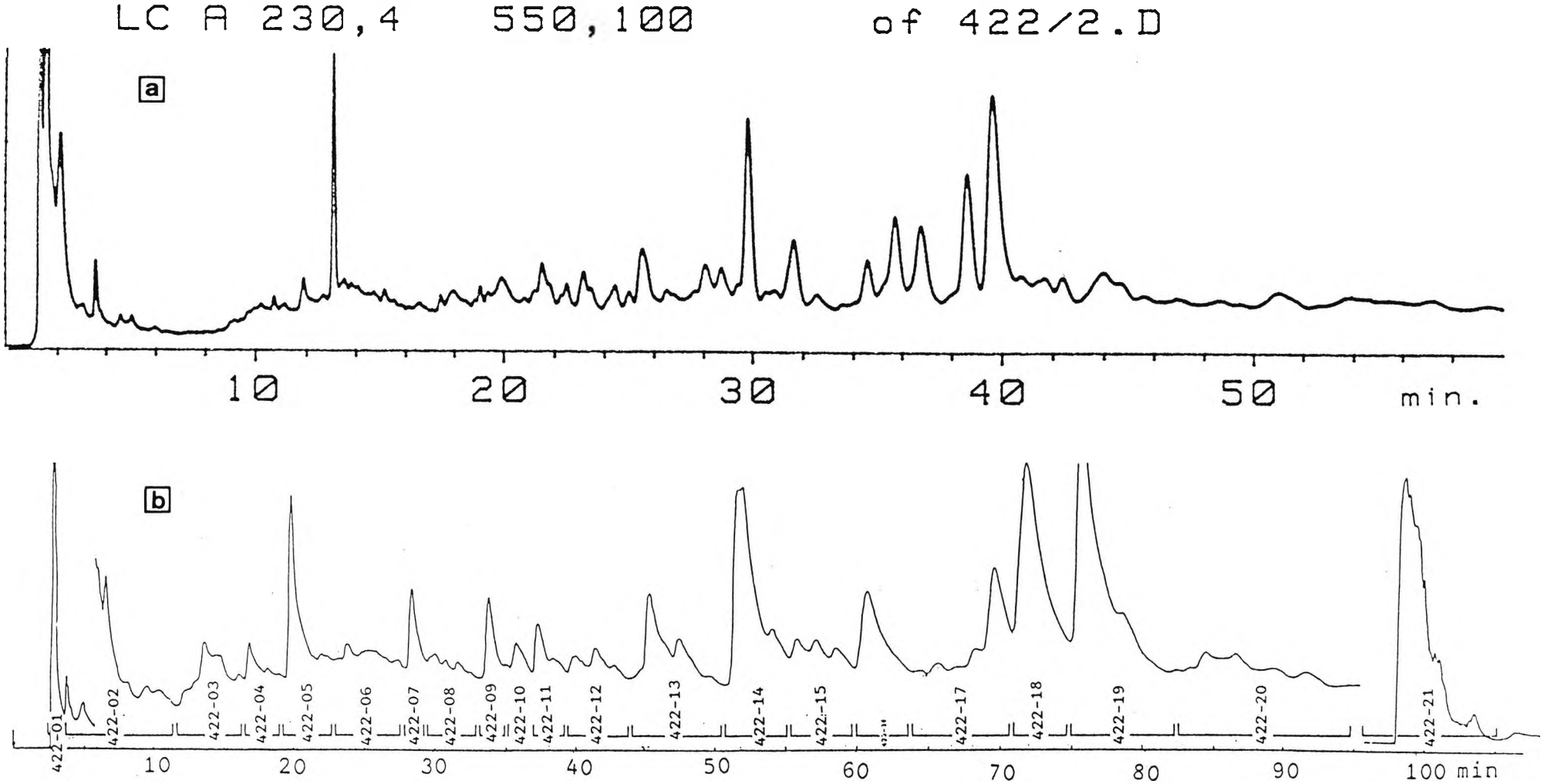


Figure 5-24. HPLC separation of TW402 by continuous gradient elution on reverse phase monitored at 230 nm. a) Analytical HPLC on 25 x 0.45 cm ODS-Hypersil 5 µm column, eluted by continuous gradient from 100% H_2O to 12:12:76 MeCN/MeOH/H_2O using a 25 ml mixing flask at 2 ml/min.
b) Preparative HPLC separation into 17 fractions on a 1" O.D. x 33 cm ODS chemically bonded to silica-Hypersil 5 µm column, eluted by continuous gradient from 100% H_2O to to 12:12:76 MeCN/MeOH/H_2O using a 1 l mixing flask at 30 ml/min, monitored at 0.02 AUFS with an analytical flowcell.

These optimum chromatographic conditions were scaled up to run the preparative (1" O.D.) column, generating a back-pressures just above 5500 psi at 30 ml/min. 750-800 mg of TW402 dissolved in warm water was loaded in a injector fitted with a 4 ml loop. Due to the very low absorbance of most of the water soluble compounds at 230 nm, an analytical 1 cm pathlengh flowcell was fitted to enhance the absorbance reading by 10; in this way the eluting compounds could be seen adequately at a sensitivity of 0.2 AUFS (Figure 5-24b).

Twenty one fractions were collected and coded 422-01 to 422-21 as shown in Figure 5-24b. The products from 5 test equivalents of TW402 were collected with an 88% recovery; after drying the subfractions under vacuum they were sent to China for testing in male rats.

Table 5-8
The effects of 422 series on the fertility of male rats.+

Group (n)	Body wt gain (g)	Weight of testis (g/Kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml	%w/w starting material	
Control(5)	114±23	1.52±0.10	84±6	89±21		
422-01(5)	109±16	1.60±0.07	87±5	84±28	8.71	
422-02(5)	106±11	1.51±0.19	88±7	96±5	7.27	
422-03(5)	125±13	1.58±0.12	91±7	111±20	10.85	
422-04(5)	146±25	1.68±0.12	92±6	69±14	11.44	
422-05(5)	138±36	1.75±0.18	90±5	67±14	22.10	
422-06(5)	146±27	1.70±0.13	89±4	73±13	30.86	
422-07(5)	145±15	1.71±0.18	87±5	67±17	0.82	
422-08(5)	141±22	1.62±0.08	83±4	68±15	0.58	
422-09(5)	126±23	1.74±0.05	84±6	69±19	0.58	
Control(5)	136±23	1.55±0.12	84±6	99±18		
422-10(5)	122±35	1.58±0.14	89±4	98±25	0.15	
422-11(5)	115±33	1.56±0.14	32±18	93±13	0.21	
422-12(5)	116±19	1.54±0.08	74±8	75±15	0.40	
422-13(5)	107±21	1.46±0.14	84±6	92±20	0.36	
422-14(5)	117±12	1.57±0.13	76±15	79±9	0.22	
422-15(5)	122±28	1.59±0.11	79±11	108±10	0.48	
422-16(5)	114±27	1.55±0.16	2.5±2.9	34±17	0.28	A
422-17(5)	137±10	1.53±0.09	2.0±2	41±14	0.41	A
422-18(5)	134±26	1.12±0.29	3.0±3	6±11	0.22	A
422-19(5)	125±19	1.56±0.10	1.0±2	28±12	0.46	A
422-20(5)	119±15	1.53±0.15	70±6	71±11	0.61	
422-21(5)	106±22	1.51±0.05	83±3	81±13	2.96	

+ Data expressed in x±SD, if applicable
A Active fraction

The antifertility test results (Table 5-8) demonstrated activity in subfractions between 422-16 and 422-19. Fraction 422-11 showed a sharp decrease in the sperm motility though the sperm density was not affected, leaving some doubt as to whether it was active. As a further check, a fraction corresponding to 422-11 was later reisolated during large scale processing of TW402 to obtain the subfraction group 422-16 to 422-19, in order to further investigate its activity.

A comparison of the chromatograms of the active subfractions 422-17, 422-18 and 422-19 and the starting material TW402 can be seen in Figure 5-25. All the major eluting peaks in the active subfractions have an UV spectrum similar to triptolide or tripdiolide.

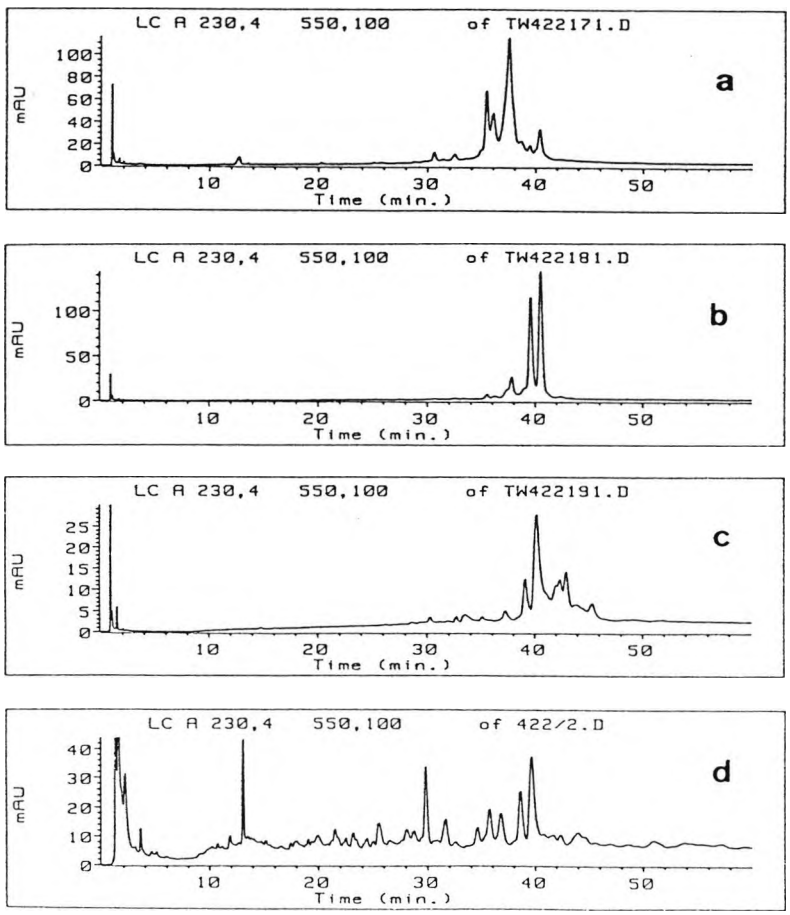


Figure 5-25. Analytical study of the active subfractions a) 422-17, b) 422-18 and c) 422-19, collected from continuous gradient elution on Hypersil-ODS preparative separation of TW402 (d). Subfractions and starting material were eluted on a 25 x 0.45 cm Hypersil-ODS 5 µm column, by continuous gradient from 100% H_2O to 12:12:76 MeCN/MeOH/H_2O using a 25 ml mixing flask at 2 ml/min, and monitored at 230 nm.

To further separate the active subfractions 422-16 to 422-19 into pure components for structural elucidation, another 5 test equivalents of TW402 were subfractionated; 6 subfractions were collected (Figure 5-26c), under the same chromatographic conditions used before and coded as in Figure 5-24b:

432F1 corresponding to 432-01 to 432-10 (inactive)
432F2 corresponding to 432-11 (only decrease sperm motility)
432F3 corresponding to 432-12 to 432-15 (inactive)
432F4 corresponding to 432-16 to 432-19 (Active)
432F5 corresponding to 432-20 (inactive)
432F6 column washed with 100% MeCN (Yellow eluent)

The active subfraction 442-F4 was compared with the starting material TW402 on analytical HPLC (Figure 5-26 b/a).

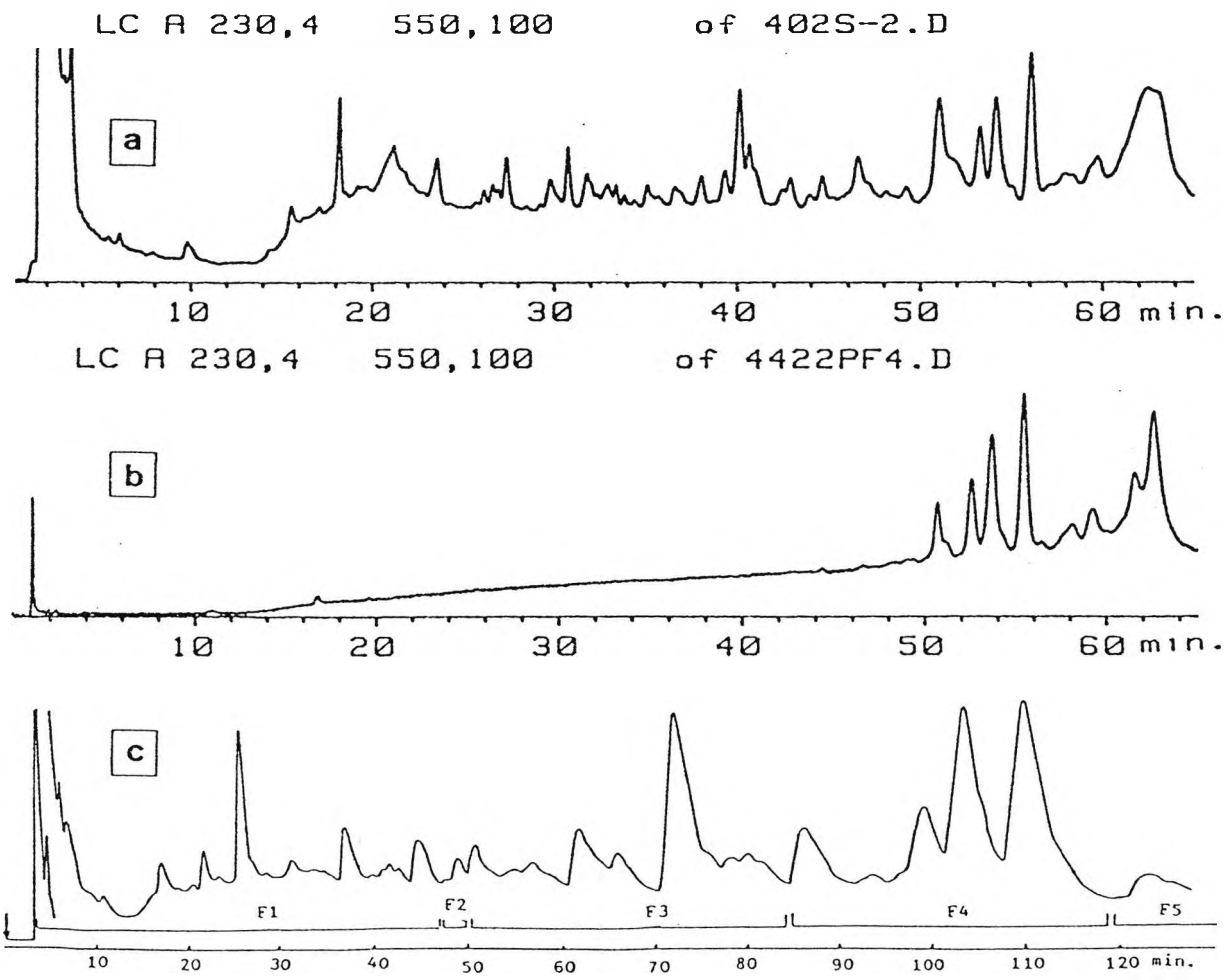
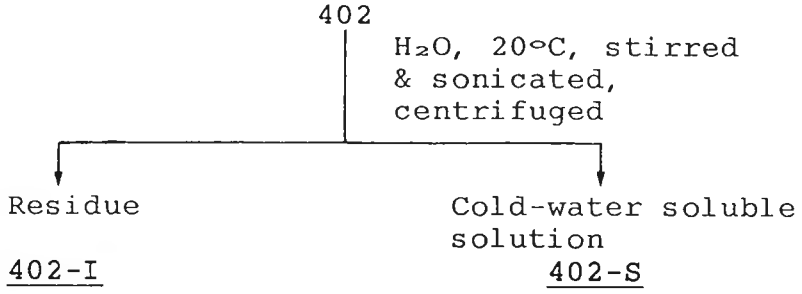


Figure 5-26. c) Preparative separation of the active subfraction 432-F4 from TW402 (chromatographic conditions as in Fig. 5-24b). Subfraction 432-F4 (b) and starting material TW402 (c) eluted on a 25 x 0.45 cm Hypersil-ODS 5 µm column by continuous gradient from 100% H_2O to 12:12:76 MeCN/MeOH/H_2O using a 25 ml mixing flask at 2 ml/min, monitored at 230 nm.

Unfortunately during this large scale HPLC subfractionation , the preparative Hypersil-ODS 5 μm column developed very high back-pressure, which could not be reduced by washing the column with strong solvents, such as methanol or acetonitrile. It was therefore suspected that an inactive excipient in TW402 may have caused the blockage of the column. Some investigation into this problem led to further solvent extraction procedure, where the inactive excipient was removed by dissolving the fraction 402 in water at ambient temperature under continuous stirring for a period of one hour (Scheme 5-2). The inactive excipient was then removed by centrifugation. This material was tested analytically under the gradient conditions. It was concluded that i) dissolution of this compound in water was extremely difficult even after prolonged warming and ii) that no compound with a chromophore above 230 nm eluted from the column when this material was injected; instead the analytical column suffered a high increase in back-pressure. By running the ambient temperature soluble sample 402-S alongside the starting material 402, both were found to contain the same eluting components; by using 402-S instead of the original sample 402, it was possible to avoid the unnecessary injection of an insoluble excipient onto the preparative HPLC column.



Scheme 5-2 Extraction sequence to remove the cold water insoluble solid from 402.

Two new preparative prepacked columns were purchased, already packed: a 50 cm x 1" O.D. Vydac-ODS 15-20 μm, 300 A and a 30 cm x 1 " O.D. Exsil-ODS 5 μm, 100 A. Both columns were protected with a 1" O.D. x 8 cm long guard column, slurry packed with the Vydac-ODS material. No change in selectivity of the eluting components from TW was found using either of the Hypersil-, Vydac- or Exsil-ODS bonded materials; comparison of Fig 5-26 and 5-27 shows similar selectivity for the elution of the active fraction 442-F4 and the starting material 402-S when using the same mobile phase on either a Hypersil-ODS or a Vydac-ODS stationary phase. Applying the same elution profile as described in Figure 5-24, 26 test equivalents were processed of 402-S on the Vydac-ODS preparative column and 6 fractions were collected and coded P1 - P6 as shown in Figure 5-27a; P5 is expected to contain the active fraction equivalent to 422-16 to 422-19. The loading was increased up to 3 g using a 13 ml sample loop. Some distortion was found in the peak shape of the eluting components with increased sample loadings and injection size, but analytical checking of the collected fractions confirmed that resolution did not deteriorate with these high loadings. Fig. 5-27b shows a comparative chromatogram of the subfraction P5 with the starting material 402-S on an analytical Vydac ODS column eluted under gradient conditions.

The animal tests results (Table 5-9) confirmed that all the antifertility action was present in the subfraction P5.

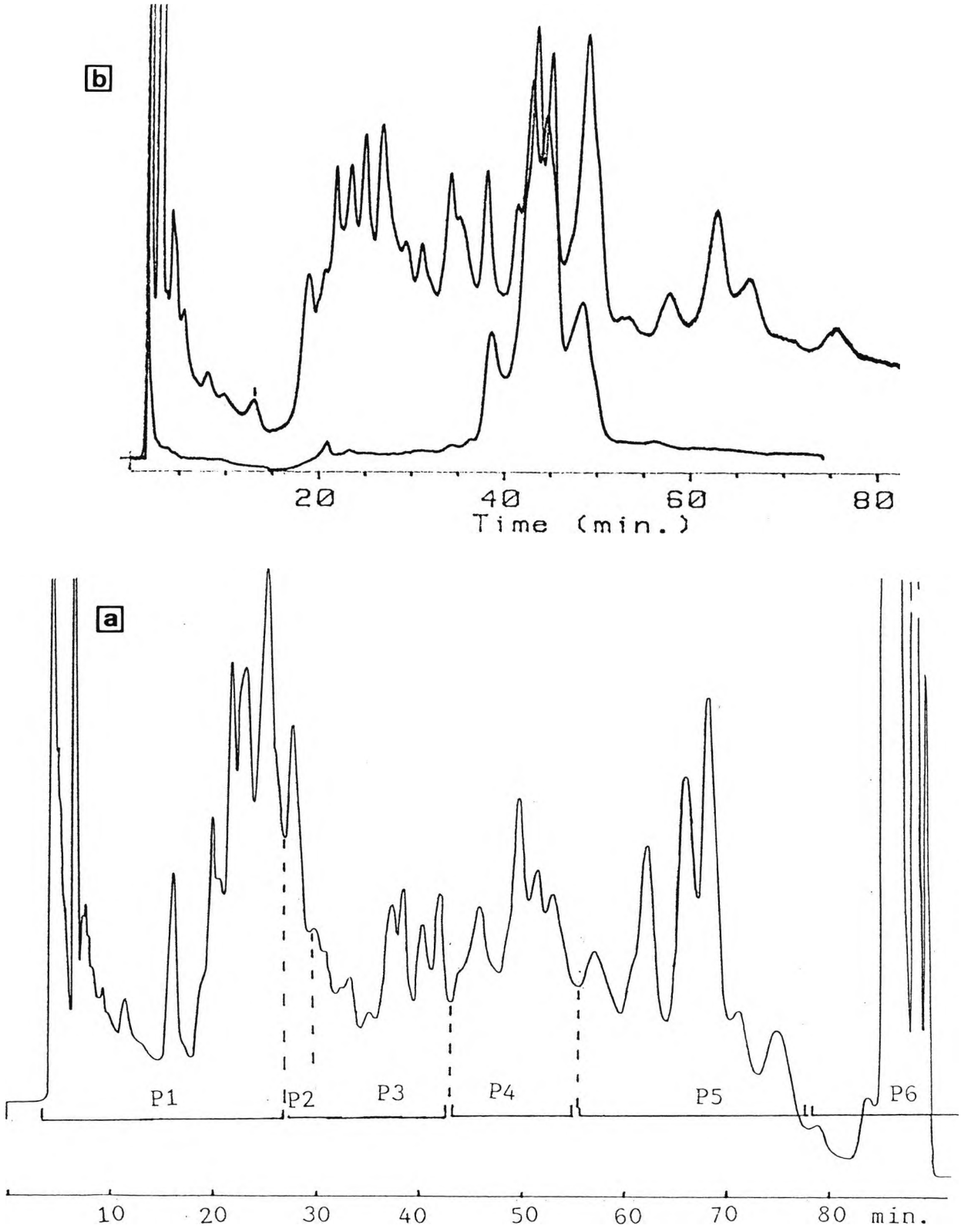


Figure 5-27. a) Separation of 402-S in 6 subfractions, on a 1" O.D. x 50 cm ODS-Vydac 300A 15-20 µm preparative column (protected with a 9 cm x 1" ODS-Vydac 300A 15-20 µm guard column), eluted for 5 min with 100% H_2O, then gradient elution to 12:12:76 MeCN/MeOH/H_2O at 36 ml/min using a 1 l mixing flask, monitored at 230 nm and 1 AUFS using an analytical flowcell. Loaded sample: up to 3 g dissolved in warm water and introduced with a 13 ml sample loop.
b) Superimposed analytical chromatograms of the active P5 and the starting material 402-S; eluted on a 25 x 0.45 cm ODS-Vydac 300A 15-20 µm, with 100% H_2O for 5 min. and then by gradient elution to 12:12:76 MeCN/MeOH/H_2O at 2 ml/min using a 25 ml mixing flask, monitored at 230 nm.

Table 5-9
The effects of Series 402 on the fertility of male rats.+

Group (n)	Body wt gain (g)	Weight of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml	% w/w starting material	
Control(5)	106±33	1.59±0.07	83±3	90±4		
442-P1(5)	98±14	1.54±0.08	82±3	86±4	30.76	
442-P2(5)	105±24	1.53±0.08	80±4	88±2	3.36	
442-P3(5)	94±28	1.63±0.16	77±3	86±4	1.92	
442-P4(5)	98±6	1.52±0.15	80±4	84±4	2.88	
442-P5(5)	102±26	1.56±0.18	1.0±2.2	45±3	2.33	A
442-P6(5)	115±3.1	1.62±0.05	78±6	86±5	1.75	

+ Data expressed in x±SD, if applicable
A Active fraction

5.5.3 P5: Isocratic separation

Isocratic chromatographic conditions containing MeOH, MeCN and water demanded very long running times, the earliest eluting components appearing after 15 mins with no baseline resolution (Figure 5-28). An optimum gradient elution based on a mobile phase composed of MeCN, MeOH and water eluted the first components just before an hour, needing over two hours for each analytical run (Figure 5-29).

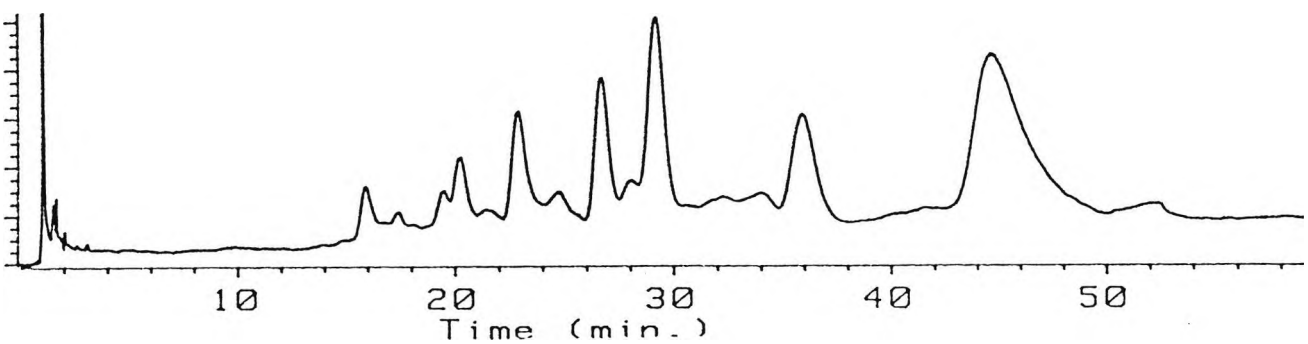


Figure 5-28. Resolution of P5 on a 25 x 0.45 cm Exsil-ODS 5 µm column, eluted with 8:8:84 MeCN/MeOH/H_2O at 2 ml/min and monitored at 230 nm.

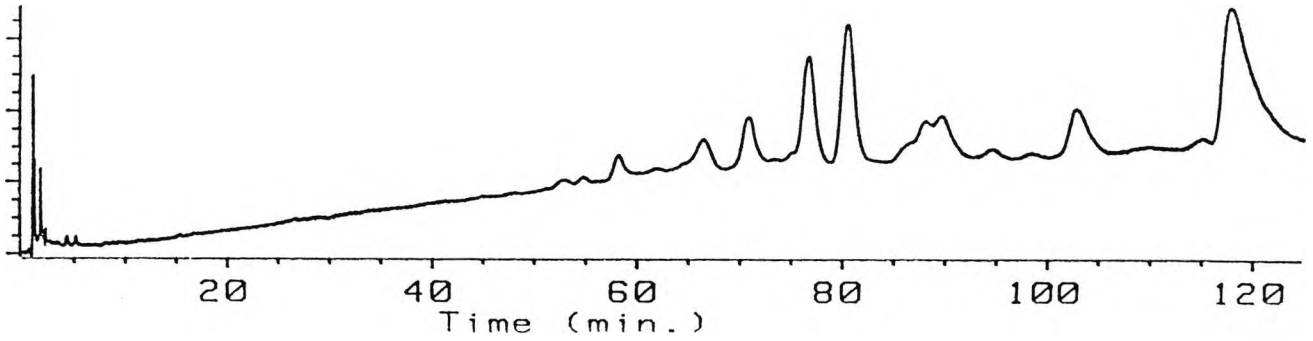


Figure 5-29. Resolution of P5 on a 25 x 0.45 cm Exsil-ODS 5 µm column, eluted with a continuous gradient from 4:4:92 to 9:9:82 MeCN/MeOH/H_2O at 2 ml/min using a 25 ml mixing flask and monitored at 230 nm.

In order to shorten the running times, a new chromatographic system containing only small proportions of THF in water was investigated. This chromatographic mobile phase gave a substantial improvement in selectivity. With this analytical system, components started to elute within 5 minutes, as well-resolved and very sharp, narrow and non-tailing peaks. Clearly, this chromatographic system could be transferred to preparative scale and good resolution could be expected. The most efficient analytical separation was obtained with a mobile phase of 4/96 THF/H_2O; a continuous gradient to 10/90 THF/H_2O was required to elute all remaining components from the column (Fig. 5-30). Four of the early eluting components presented UV spectra similar to that of triptolide. Because the mobile phase applied in this case is very polar, these analogues of triptolide had to be also much more polar than triptolide itself. An available sample of tripdiolide obtained from Dr Kutney was run under the same chromatographic conditions. The first and largest peak eluting in the P5 mixture proved to be tripdiolide.

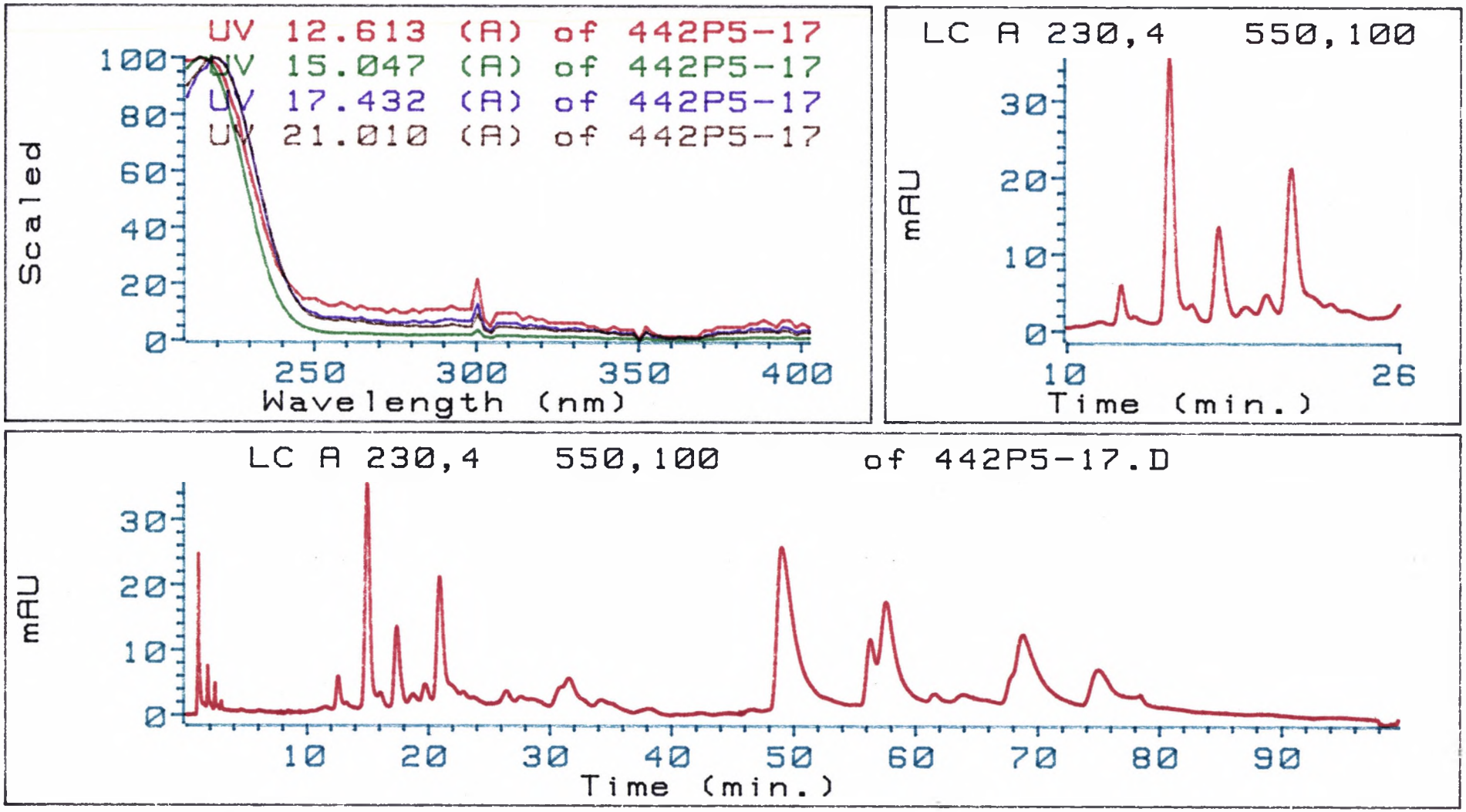


Figure 5-30. HPLC separation of the active preparative subfraction P5 (bottom) and close up section of the chromatogram containing the 4 early eluting peaks (top right), all with UV spectra superimposable to that of triptolide (top left), eluted on a 25 x 0.45 cm Exsil-ODS 5 µm column, with 96/4 H_2O:THF for 20 min and gradient elution to 90/10 H_2O:THF at 2 ml/min, using a 25 ml mixing flask, and monitored at 230 nm with a HP 1040 photodiode array detector, using a 20 µl sample loop.

Initially, the chromatographic conditions for the preparative separation on HPLC were transferred from the analytical system previously described (Figure 5-31c) to an efficient preparative Exsil-ODS 5 um column protected with a precolumn containing a coarser ODS-Vydac packing material (Figure 5-31a). A reasonably good separation of the early eluting compounds was achieved, well resolved components were collected separately and the unresolved fractions and inter-peak regions were pooled together and subsequently rechromatographed with a weaker 2/98 THF/H_2O mobile phase (Figure 5-31b). These procedures provided a total of 19 fractions, coded P7 - P25. .

Four fraction P8, P12, P13 and P16 which were all obtained with good level purity, showed the same UV spectra as can be seen in Figures 5-32 to 5-35, respectively. Fraction P12 was already identified as tripdiolide by comparison with the reference standard obtained from Dr Kutney. The other three fractions showing the same UV spectra as triptolide or tripdiolide, appeared to be their derivatives. All these four fractions were sent to Dr. J.K.M. Sanders for spectroscopic analysis.

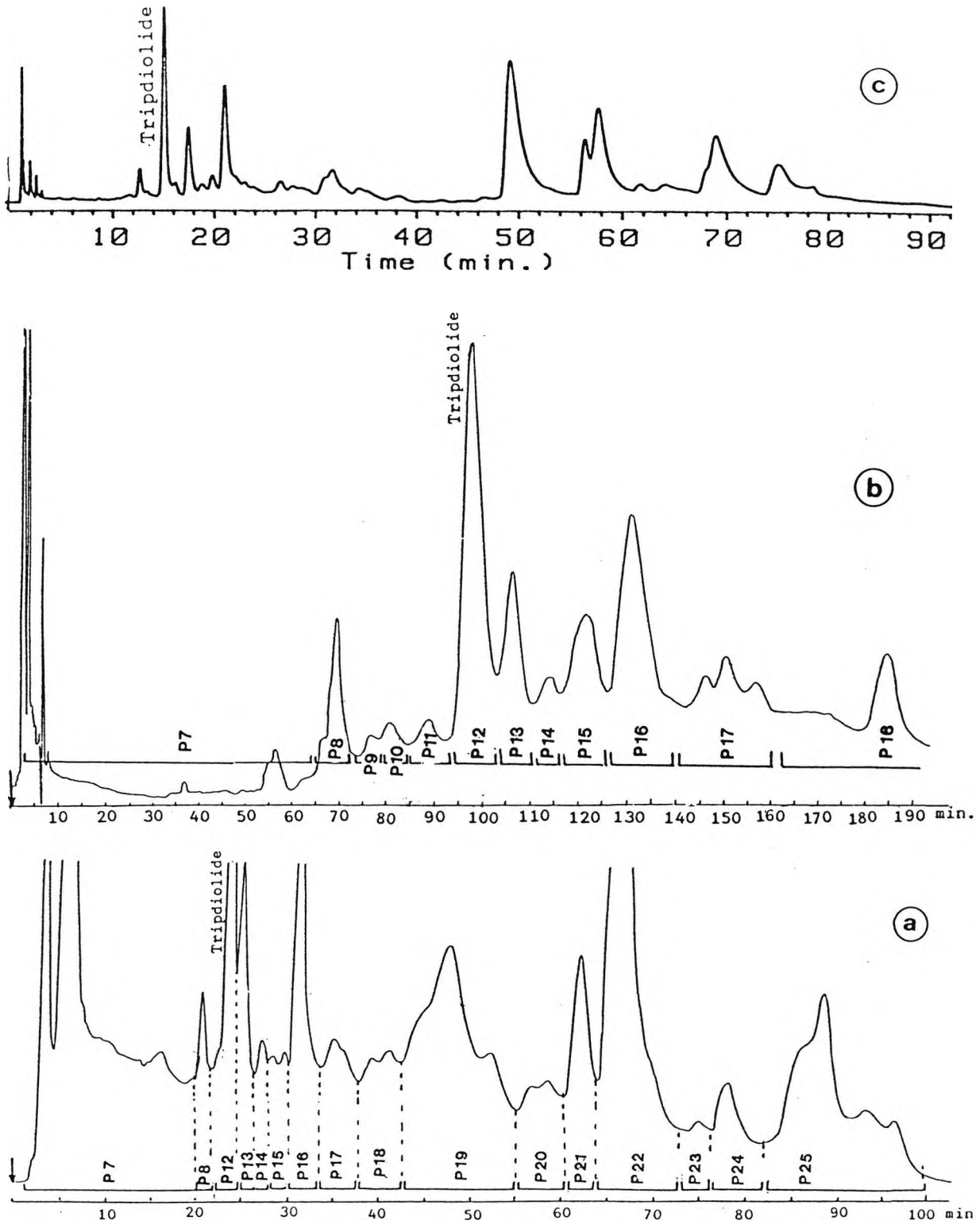


Figure 5-31. Preparative HPLC separation of the active fraction P5, on a 1" O.D. x 40 cm ODS-Exsil 5 μm preparative column (protected with 1" O.D. x 8 cm ODS-Vydac 300Å 15-20 μm guard column), and eluted with 96:4 H_2O/THF for 50 min. and then gradient elution to 90/10 H_2O/THF at 25 ml/min using a 1 litre mixing flask, 100 mg sample was loaded on a 1 ml injection loop (A), previous unresolved preparative fractions and the interpeak regions are pooled together and rechromatographed at 98:2 H_2O/THF at 30 ml/min (B), monitored at 230 nm and 0.02 AUFS using an analytical flowcell.
C) Analysis of P5 under the conditions of Figure 5-30.

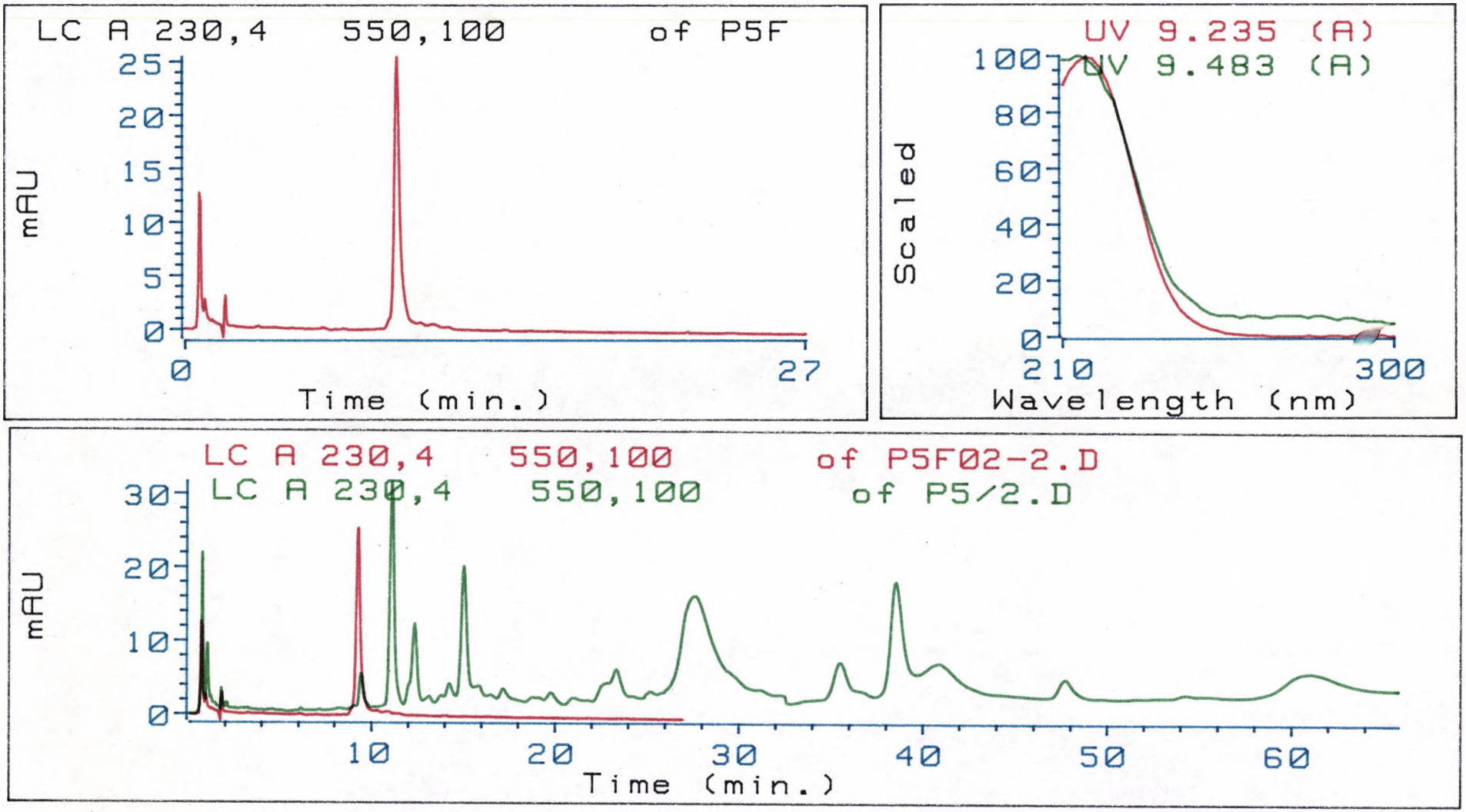


Figure 5-32. **Analysis of the preparative subfraction P8 (top left)** and superimposed chromatograms of P8 and the starting material P5 **(bottom)** on a 25 x 0.45 cm Exsil-ODS 5 µm column, eluted with 96/4 H_2O:THF at 2 ml/min and monitored at 230 nm with a HP 1040 photodiode array detector, using a 20 µl sample loop. **Top right:** Superimposed UV spectra of P8 and that of the peak corresponding to P8 in the starting material P5.

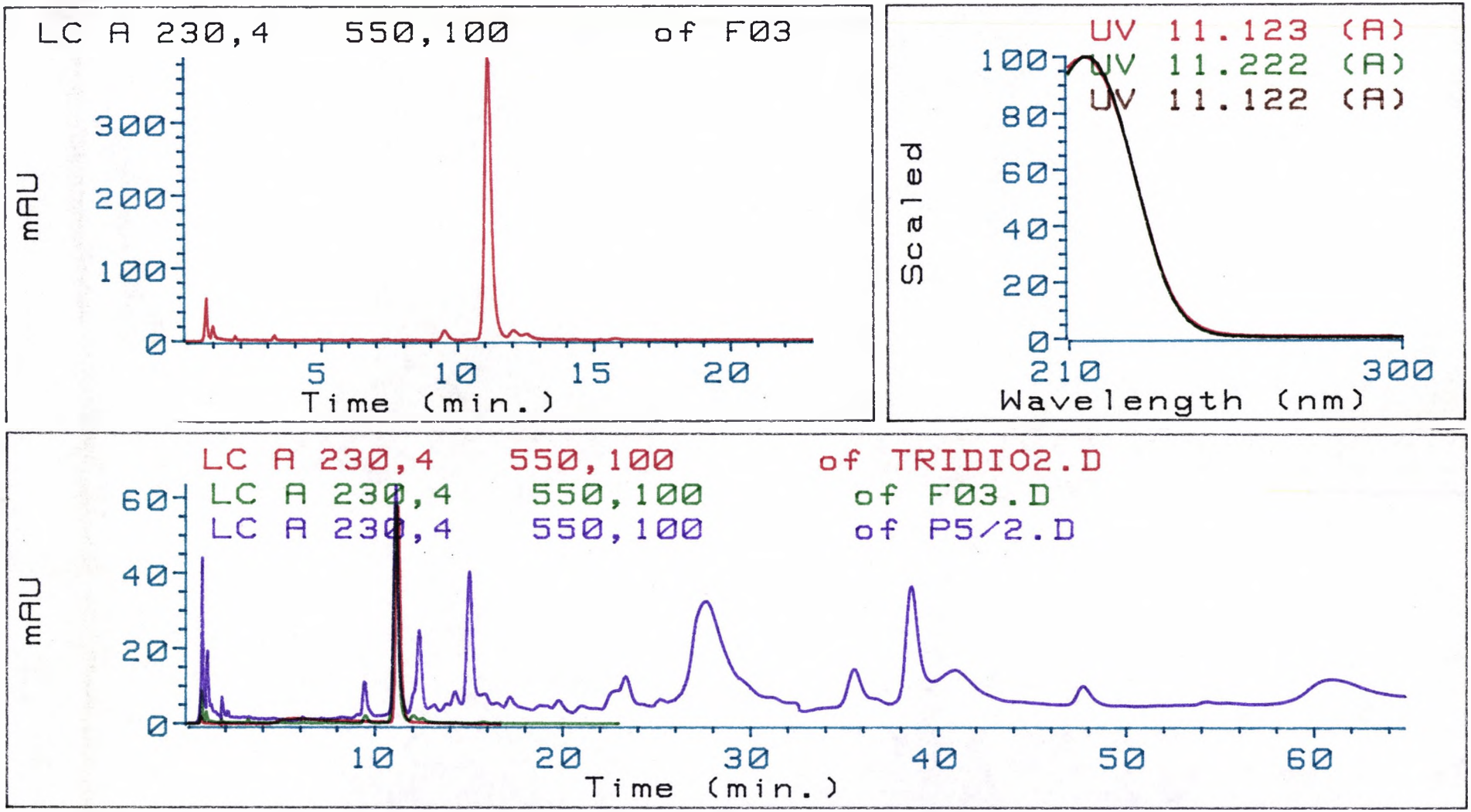


Figure 5-33. Analysis of the preparative subfraction P12 (top left), and superimposed chromatograms of P12, the starting material P5 and a reference sample of **tripdiolide (bottom)** on a 25 x 0.45 cm Exsil-ODS 5 µm column, eluted with 96/4 H_2O:THF at 2 ml/min and monitored at 230 nm with a HP 1040 photodiode array detector, using a 20 µl sample loop. **Top right:** Superimposed UV spectra of P12, that of the peak corresponding to P12 in the starting material P5 and a reference sample of tripdiolide. Obviously, P12 is a reasonable sample of tripdiolide..

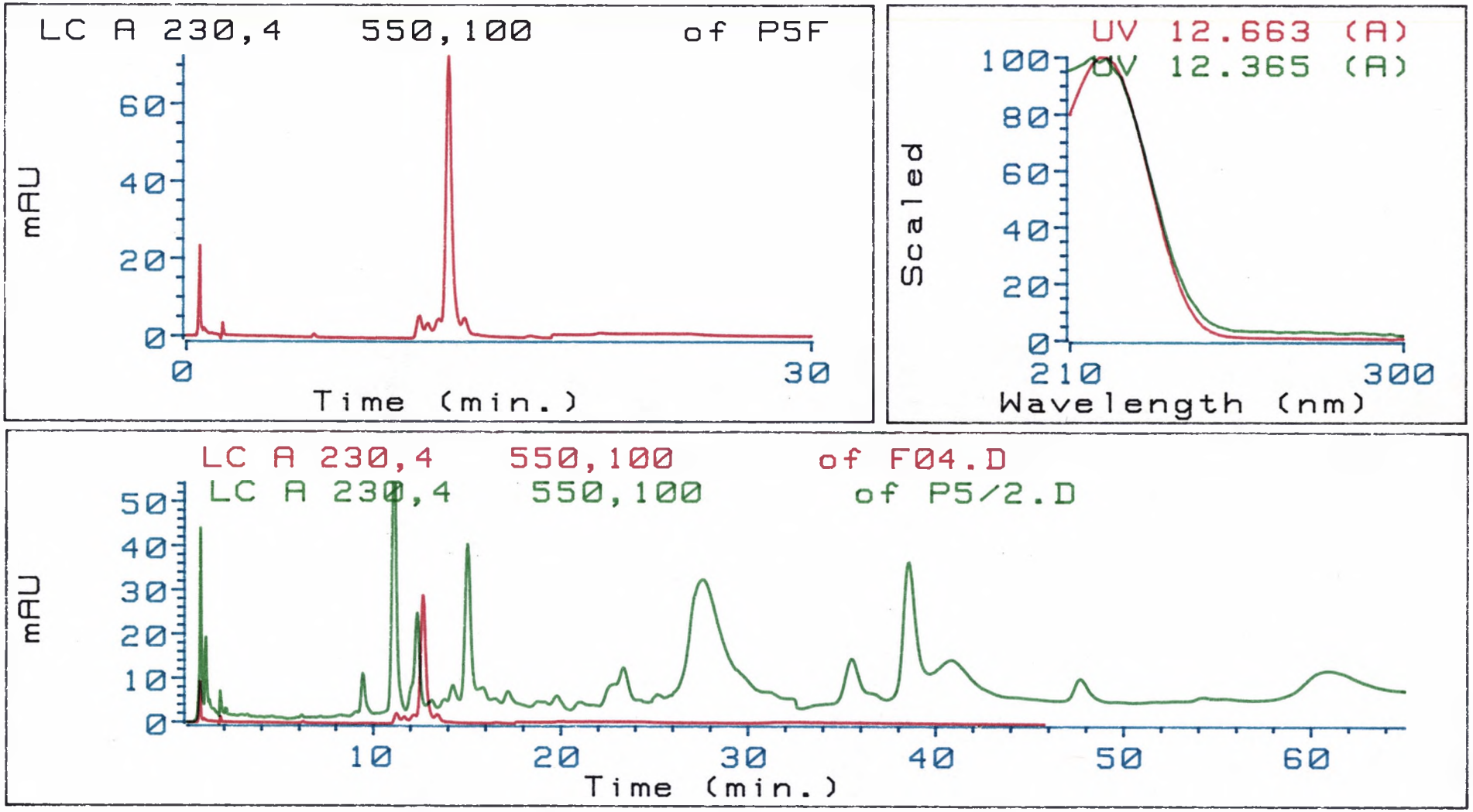


Figure 5-34. Analysis of the preparative subfraction P13 (top left) and superimposed chromatograms of P13 and the starting material P5 (bottom) on a 25 x 0.45 cm Exsil-ODS 5 µm column, eluted with 96/4 H_2O:THF at 2 ml/min and monitored at 230 nm with a HP 1040 photodiode array detector, using a 20 µl sample loop. **Top right:** Superimposed UV spectra of P13 and that of the peak corresponding to P13 in the starting material P5.

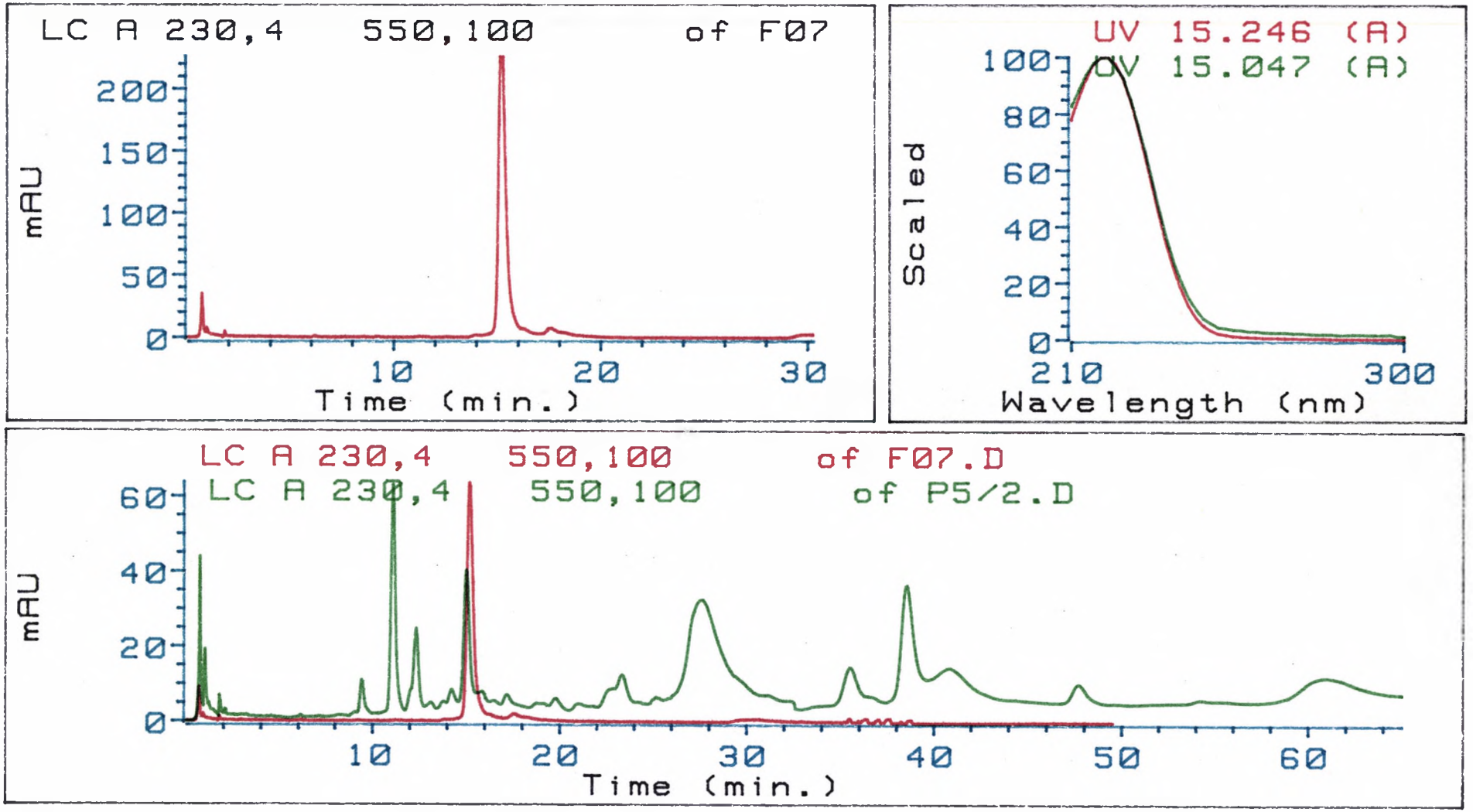


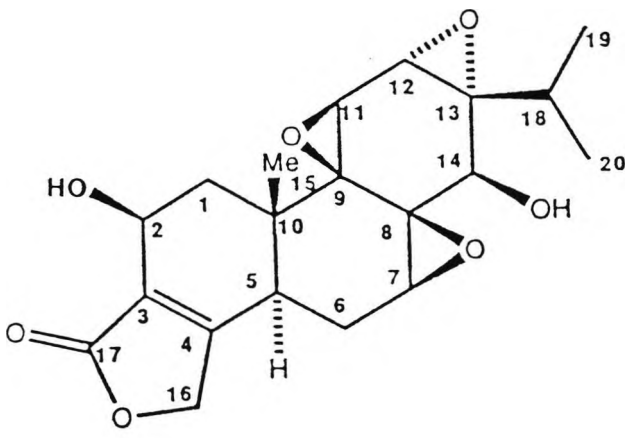
Figure 5-35. Analysis of the preparative subfraction P16 (top left) and superimposed chromatograms of P16 and the starting material P5 (bottom) on a 25 x 0.45 cm Exsil-ODS 5 µm column, eluted with 96/4 H_2O:THF at 2 ml/min and monitored at 230 nm with a HP 1040 photodiode array detector, using a 20 µl sample loop. **Top right:** Superimposed UV spectra of P16 and that of the peak corresponding to P16 in the starting material P5.

5.5.4 Structural elucidation of P8, P12, P13 and P16

Within the 19 subfractions, numbers P12, P13 and P16 were considered to be of particular importance, since they corresponded to three of the most prominent components present in the active grouping 422-16 to 422-19 analysed earlier. The following results were obtained (thanks, in particular to Dr. J.K.M. Sanders in Cambridge, who assisted us with high field NMR spectra).

P12

This sample was found to be virtually 100% pure by HPLC (Fig. 5-33) and identical (HPLC and high field NMR, Fig. 5-36) to the sample of tripdiolide (4)provided by Dr. Kutney. We were also informed by Dr. Qian that a pure sample of tripdiolide from Dr. Kutney had proven to be active in the antifertility assay. The structural assignment is fully supported by the ^{1}H NMR spectrum, which is in total agreement with the data published by Kupchan[1-3] and Kutney[4], and results of nOe difference spectroscopy (Table 5-10) and ^{13}C NMR spectrum (Table 5-11). The couplings shown in Table 5-10 were established using COSY.



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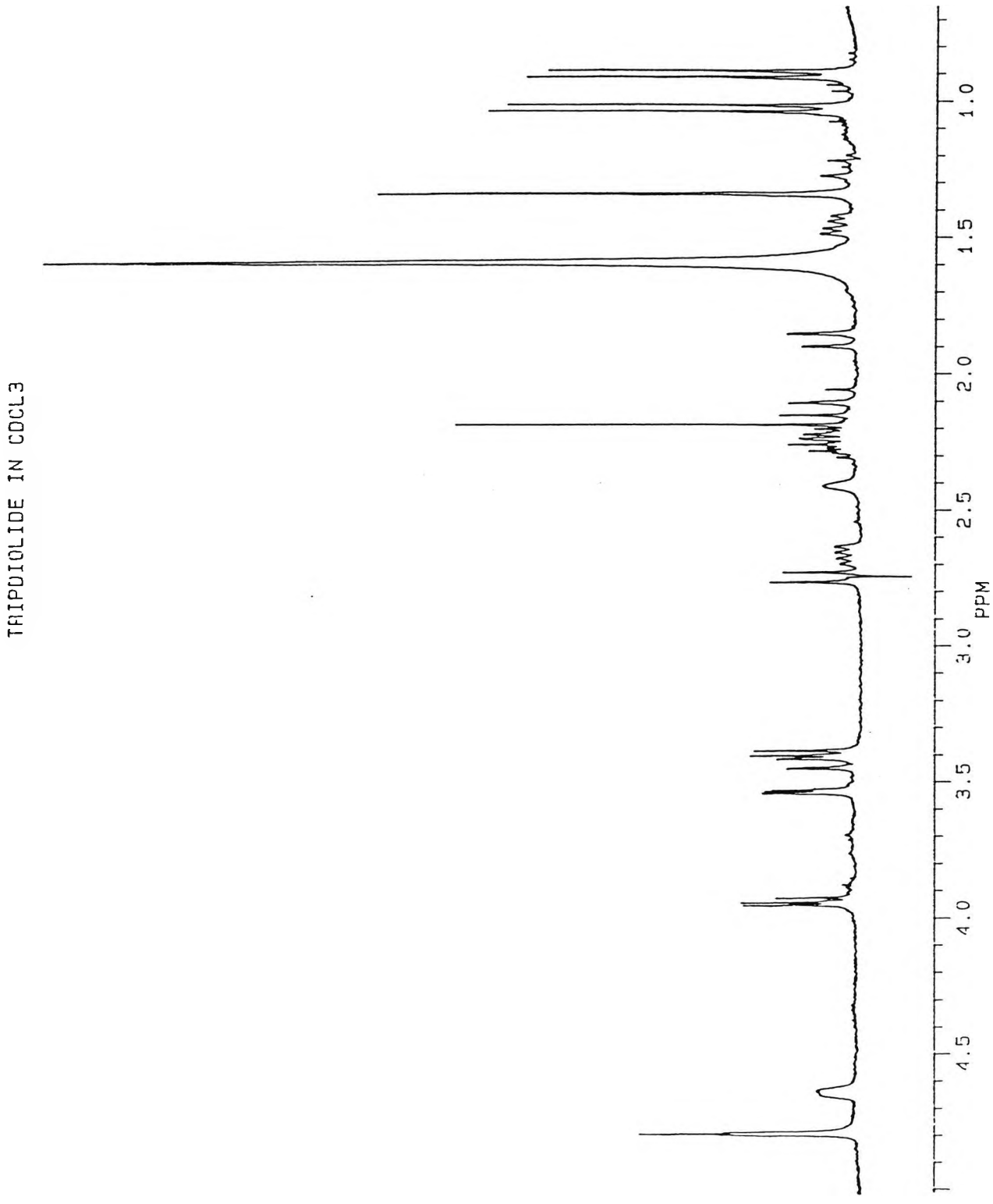


Figure 5-36: ^{1}H NMR of tripdiolide (Fraction P12)

^{1}H data ($CDCl_3$) TRIPDIOLIDE (P12)

Proton	Chemical Shift (ppm)	Multiplicity & coupling (H3)	Protons connected by scalar coupling
1 ax	1.45	ddm (14.0, 6.2)	1 eq 2 15
1 eq	1.88	dd (14.0, 1.0)	1 ax 2
2	4.63	dm (6.2)	1 ax 16 2-OH 1 eq
2-OH	2.39	bm	2
5	2.66	ddm (13.2, 6.5)	6 ax 6eq 16
6 ax	2.10	dd (14.7, 12.9)	6 eq 5
6 eq	2.24	ddd(14.7, 6.5, 5.4)	6 ax 5 7
7	3.38	d (5.4)	6 eq
11	3.94	d (3.4)	12
12	3.53	dd (3.4, 1.0)	11 14
14	3.43	dd (10.6, 1.0)	14-OH 12
14-OH	2.74	d (10.6)	14
15	1.34	d (1.0)	1 ax
16	4.78	dd (1.3, 1.0)	5 2
18	2.25	sep (7.0)	19 20
19	1.12	d (7.0)	18 20
20	0.90	d (7.0)	18 19

nOe difference experiments

Proton irrad.	1 ax	1 eq	2	2 OH	5	6 ax	6 eq	7	11	12	14 OH	14	15	16	18	19	20
1ax		s	s						s								
2	s	w															
6ax					m		s										
11	s	w								s							
15		m							w								

s = strong, m = medium, w = weak

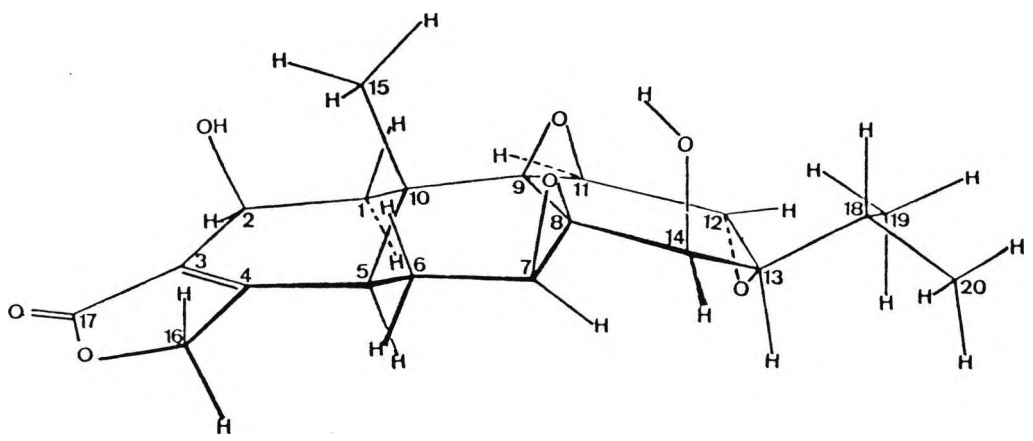


Table 5-10 : 1H NMR and nOe experiments on fraction P12 (Tripdiolide)

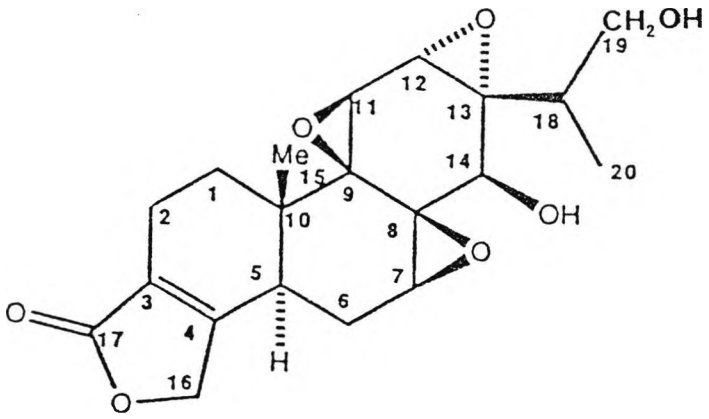
Table 5-11: ^{13}C Data ($CDCl_3$) TRIPDIOLIDE (P12)

Carbon	Chemical shift (ppm)		
1	28.22		
2	59.41		
3	161.85		
4	126.97		
5	40.79		
6	23.32		
7, 11, 12*	59, 96	57.47	54.55
8, 9, 13*	66.31	65.74	60.88
10	35.94		
14	73.48		
15	15.29		
16	70.07		
17	178.97		
18	38.07		
19	17.73		
20	16.86		

* Assigments uncertain within groups of signals.

P13

This sample was found to be almost pure by HPLC (Figure 5-34) and NMR. The high field ^{1}H-NMR spectrum (Figure 5-37, Table 5-12) was virtually superimposable on that of triptolide, but had only one methyl group (3H, doublet) instead of two in the isopropyl region and a 1H quartet of double doublets (δ 2.28) replacing the septet of an isopropyl methine. This data indicated that alteration to one of the isopropyl methyl groups of triptolide has occurred, as supported by the ^{13}C NMR spectrum (Table 5-13) and particularly by the chemical shift found at C-19 indicative of substitution by an oxygen. Thus, the compound was assigned the structure of 19-hydroxytriptolide (5), i.e. it is an isomer of tripdiolide, not previously reported in the literature. This assignment is supported by the mass spectrum of the compound, which shows the expected, weak molecular ion at m/z 376 ($C_{20}H_{24}O_7$) in the EI mode. In FAB mode, the mass spectrum gave ions at m/z 377 (M+H) and m/z 399 (M+Na) as would be expected.



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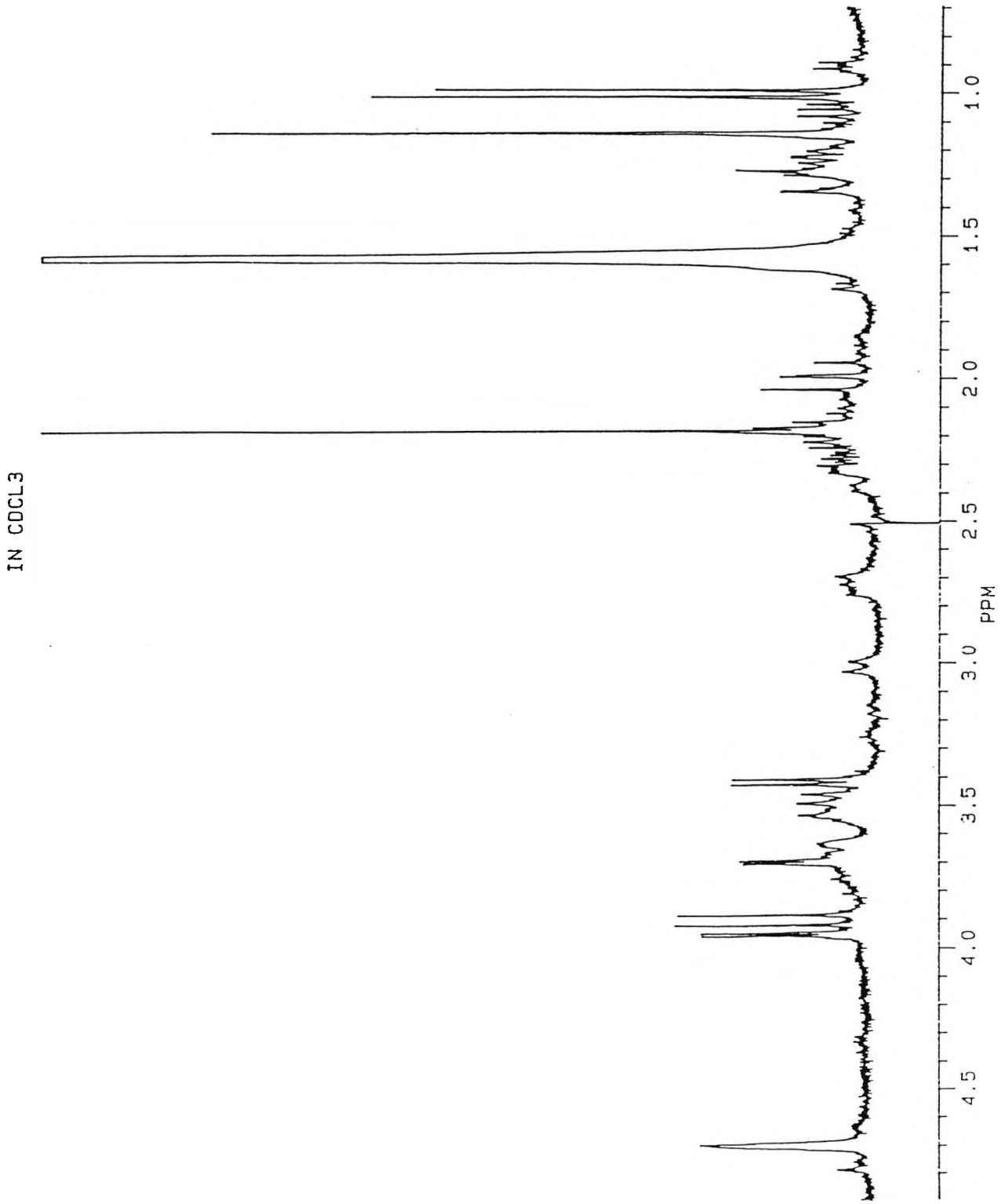


Figure 5-37: ^{1}H NMR of 19-hydroxytriptolide (Fraction P13)

^{1}H data ($CDCl_3$) 19-Hydroxytriptolide P13

Proton	Chemical Shift (ppm)	Multiplicity & coupling (H3)	Protons connected by scalar coupling
1 ax	1.24 obs.	dddm (12.9, 6.5, 4.5)	1eq 2ax 2eq 15
1 eq	1.61 obs.	ddm (12.9, 4.5)	1 ax 2ax 2eq
2 ax	2.13 obs.	dm (17.9	2eq 1ax 1eq 16
2 eq	2.35	dm (17.9)	2ax 1ax 1eq 16
5	2.72	m	6 ax 6eq 16
6 ax	1.99	dd (15.1, 13.7)	6 eq 5
6 eq	2.20	ddd(15.1, 6.5, 5.3)	6 ax 5 7
7	3.41	d (5.3)	6 eq 6ax
11	3.95	d (3.2)	12
12	3.70	dd (3.2, 1.0)	11 14
14	3.47	d (10.1)	14-OH
14-OH	3.00	d (10.1)	14
15	1.14	d (1.0)	1 ax
16	4.69	m	2ax 2eq 5
18	2.28 obs.	qdd (7.2, 4, 4)	19 19′ 20
19	3.65 obs.	dt (12.0, 4)	19′ 18 19-OH
19′	3.53 obs.	dt (12.0, 4)	19 18 19-OH
20	1.00	d (7.2)	18

nOe difference experiments

proton irrad.	1 ax	1 eq	2 ax	2 eq	5	6 ax	6 eq	7	11	12	14	14 OH	15	16	18	19	19′	20
2eq			m															
5								w						w				
6ax								s	w				s					
18/2eq/ 6eq(2ax)						w		w		w	w		m	w		m	m	m
19(12)									w						s			m
19′ (7)						w	m									w	w	w
20										m	m				m	m	m	

s = strong, m = medium, w = weak

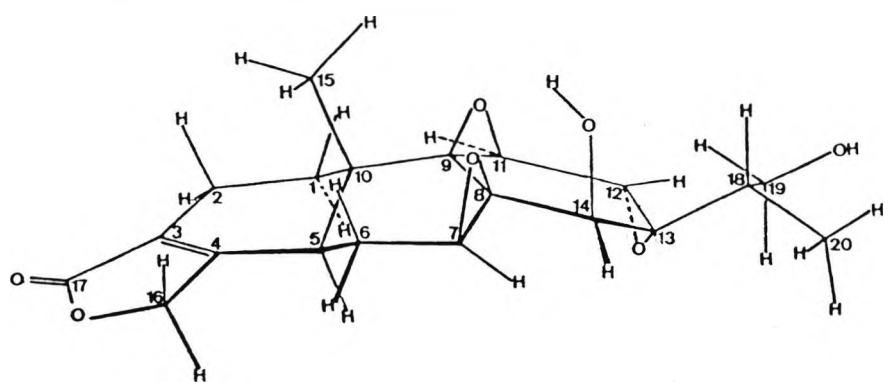


Table 5-12 : 1H NMR and nOe experiments on fraction P13 (19-hydroxytriptolide)

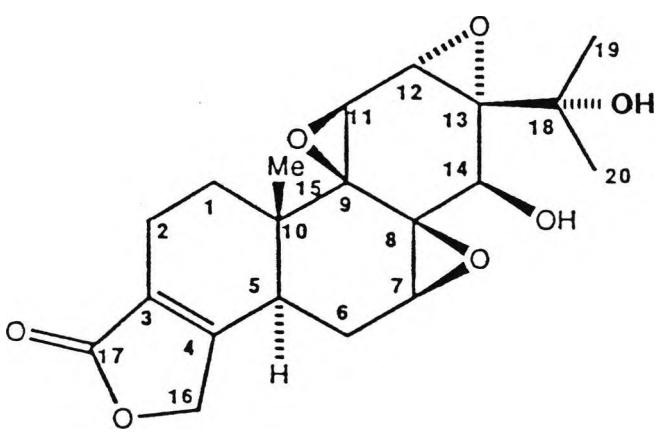
Table 5-13: ^{13}C Data ($CDCl_3$) 19-HYDROXYTRIPTOLIDE (P13)

Carbon	Chemical shift (ppm)		
1	17.07		
2	29.83		
3	159.73		
4	125.64		
5	40.47		
6	23.66		
7, 11, 12*	60,09	56.72	54.35
8, 9, 13*	66.38	64.04	60.59
10	35.82		
14	74.17		
15	12.32		
16	69.91		
17	Not seen		
18	37.25		
19	64.04		
20	13.63		

* Assigments uncertain within groups of signals.

P16

This sample was found to be almost pure by HPLC (Fig. 5-35)and NMR. The high field ^{1}H NMR spectrum (Figure 5-38, Table 5-14) was extremely similar to that of triptolide, but showed differences in the isopropyl region (2 Me singlets, no methine) consistent with there having been an oxidation of $-Me_2C-H$ to $-Me_2C-OH$. Thus, this compound is assigned the structure 18-hydroxytriptolide (6), an isomer of tripdiolide previously reported by the Chinese and named triptolidenol.[6] This structure is also supported by the ^{13}C NMR spectrum (Table 5-15) which is fully consistent with the large chemical shift (70.02) found at the C-18 indicative of substitution by an oxygen, and by the mass spectrum, which shows the expected, weak molecular ion at m/z 376 ($C_{20}H_{24}O_7$) in EI mode. In FAB mode, the mass spectrum gave ions at m/z 377 (M+H) and m/z 399 (M+Na) as would be expected. The nOe difference spectra (Table 5-14) are also fully in agreement with the proposed structure.



(6)

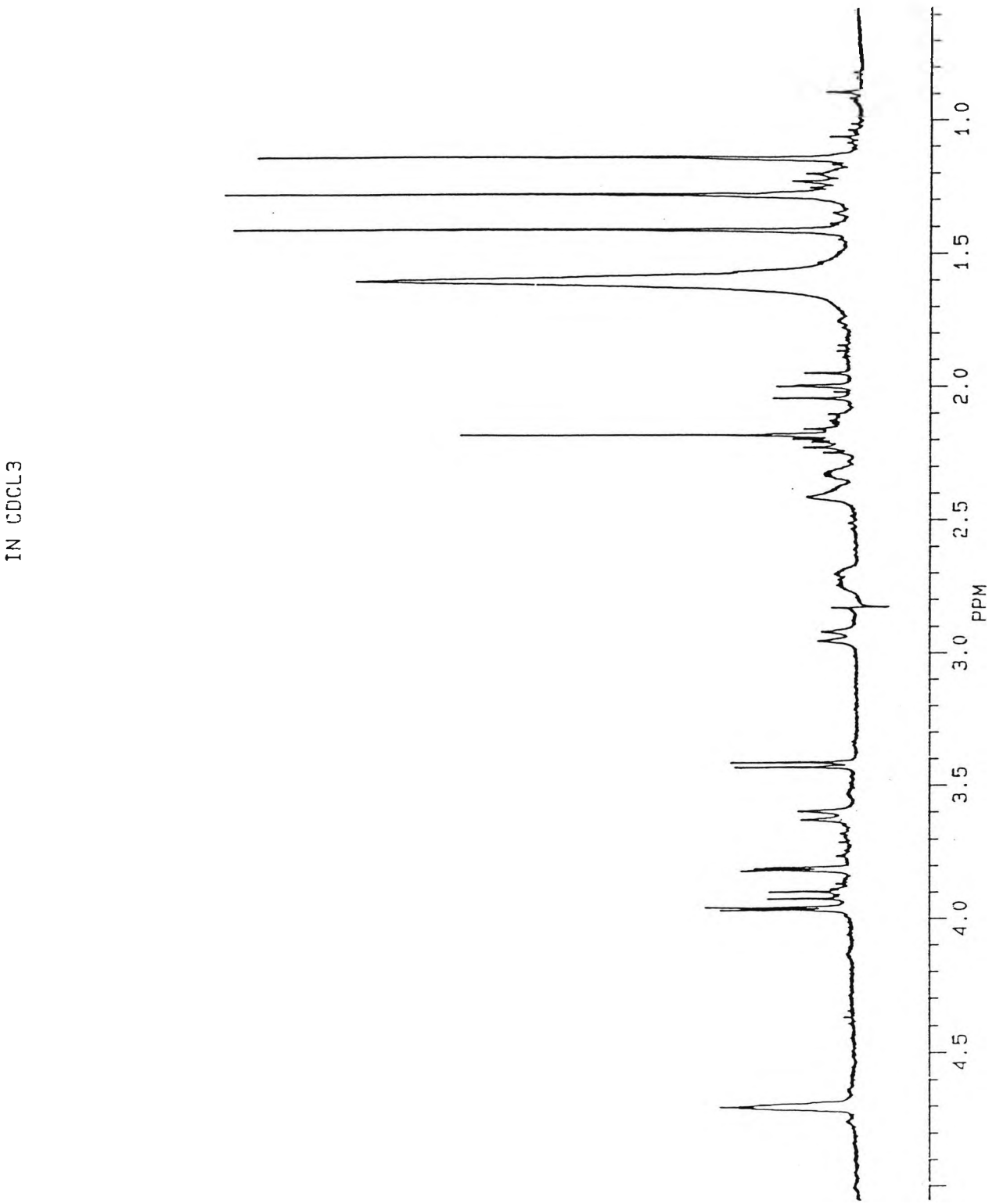


Figure 5-38: ^{1}H NMR of triptolidenol (Fraction P16)

^{1}H data ($CDCl_3$) TRIPTOLIDENOL P16

Proton	Chemical Shift (ppm)	Multiplicity & coupling (H3)	Protons connected by scalar coupling
1 ax	1.23 obs.	dddm (12.6, 6.2, 4.7)	1eq 2ax 2eq 15
1 eq	1.60 obs.	ddm (12.6, 4.7)	1 ax 2ax 2eq
2 ax	2.16 obs.	dm (18.0)	2eq 1ax 1eq 16
2 eq	2.35	dm (18.0)	2ax 1ax 1eq 16
5	2.72	dm (13.3)	6 ax 6eq 16
6 ax	1.99	dd (14.7, 13.3)	6 eq 5
6 eq	2.20	ddd(14.7, 6.4, 5.5)	6 ax 5 7
7	3.41	d (5.5)	6 eq 6ax
11	3.95	d (3.2)	12
12	3.80	dd (3.2, 1.0)	11 14
14	3.61	dd (10.0, 1.0)	14-OH 12
14-OH	2.93	bd (10.0)	14
15	1.14	s	1 ax
16	4.70	m	2ax 2eq 5
18-OH	2.41	bs	
19	1.41	s	20
20	1.23	s	19

nOe difference experiments

Proton irrad.	1 ax	1 eq	2 ax	2 eq	5	6 ax	6 eq	7	11	12	14	14 OH	15	16	18 OH	19	20
1ax/ 20		m		w					m	s	s						
7						w	m				s						
11	m									s							
12									m								
15	w	w				m											
19										m	m						

s = strong, m = medium, w = weak

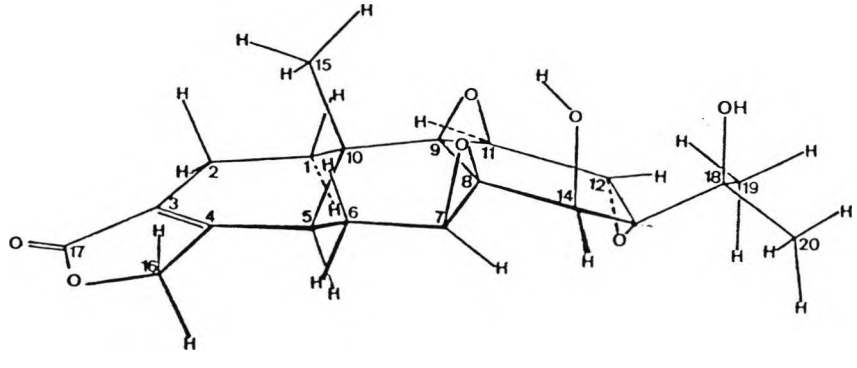


Table 5-14 : 1H NMR and nOe experiments on fraction P16 (Triptolidenol)

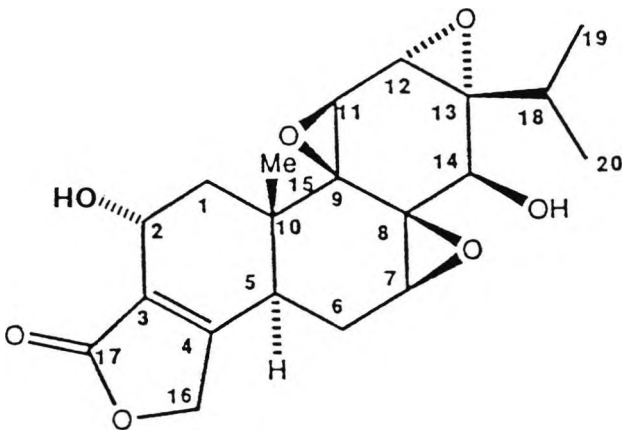
Table 5-15: ^{13}C Data ($CDCl_3$) TRIPTOLIDENOL (P16)

Carbon	Chemical shift (ppm)		
1	17.07		
2	29.82		
3	150.65		
4	125.68		
5	40.47		
6	23.67		
7, 11, 12*	60, 35	56.16	53.71
8, 9, 13*	66.10	65.63	60.89
10	35.84		
14	73.08		
15	13.63		
16	69.90		
17	173.15		
18	70.02		
19	26.53		
20	25.68		

* Assigments uncertain within groups of signals.

P8

The recognition of the presence of the three isomeric hydroxy-derivatives above in the active subfraction encouraged us to focus attention on a further peak, eluting just before tripdiolide, which had a similar UV spectrum (Fig. 5-32). This material, fraction P8, was also submitted to spectroscopic examination. The high field ^{1}H NMR (Figure 5-39, Table 5-16) shows considerable similarity to that of tripdiolide and analysis of the data (Table 5-16) supports the conclusion that this is the 2-alpha epimer of tripdiolide, a compound reported in Chinese literature[7] and named tripterolide (7). The nOe (Table 5-16) from 2β-H to the CH_3 protons (H15) is particularly indicative of the 2α-OH orientation in compound P8. This structure is also supported by the ^{13}C NMR spectrum (Table 5-17).



(7)

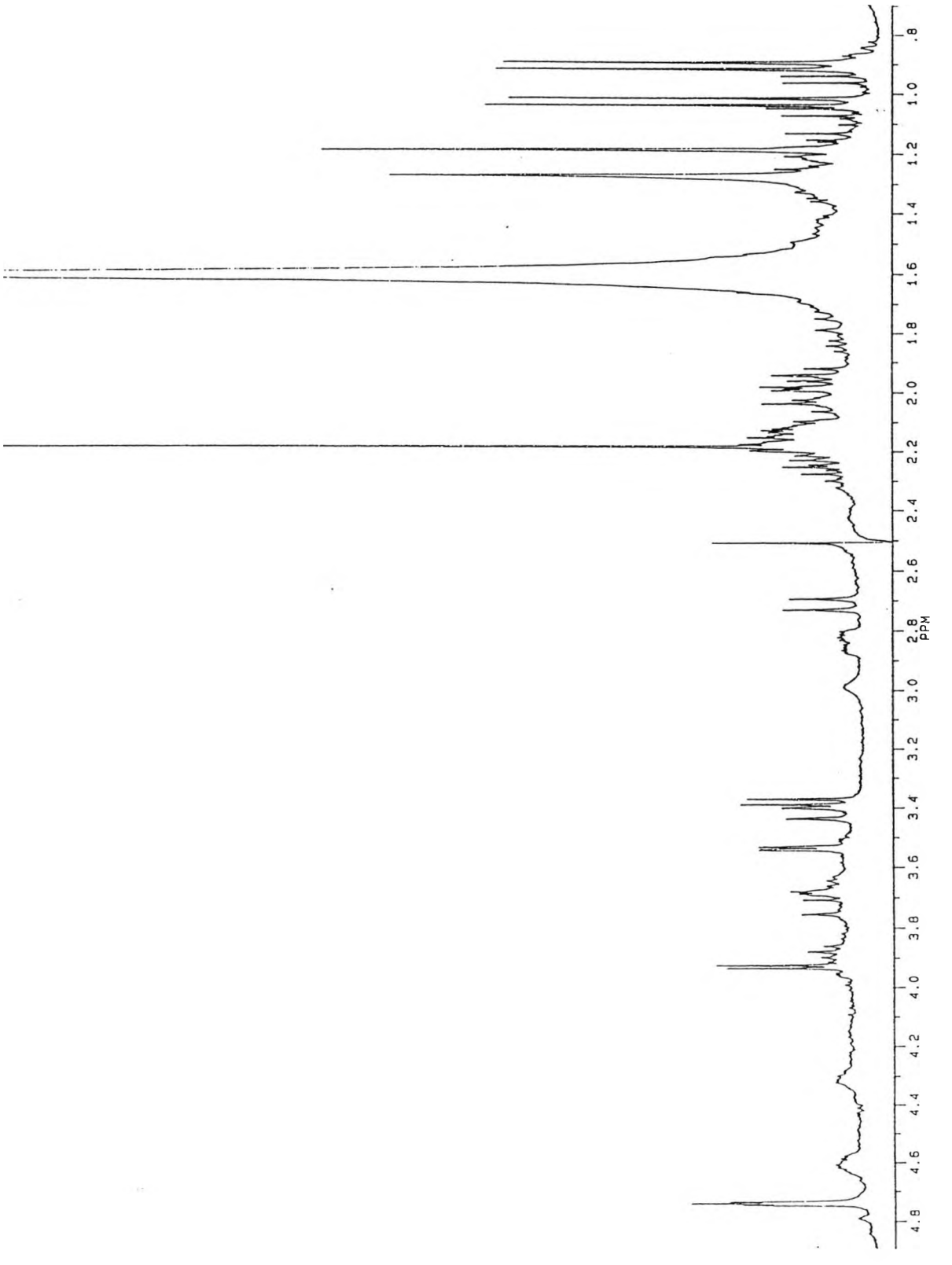


Figure 5-39: ^{1}H NMR of tripterolide (Fraction P8)

^{1}H data ($CDCl_3$) TRIPTEROLIDE (P8)

Proton	Chemical Shift (ppm)	Multiplicity & coupling (H3)	Protons connected by scalar coupling
1 ax	1.22 obs.	dd (12.1, 8.8)	1 eq 2 15
1 eq	1.95	dd (12.1, 6.1)	1 ax 2
2	4.60	bm	1 ax 1 eq
2-OH	2.98	bm	
5	2.84	ddm(13.0, 4.9)	6 ax 6eq 16
6 ax	1.99	dd (14.7, 13.0)	6 eq 5
6 eq	2.22	ddd(14.7, 5.5, 4.9)	6 ax 5 7
7	3.38	d (5.5)	6 eq
11	3.93	d (3.2)	12
12	3.53	dd (3.2, 1.0)	11 14
14	3.42	dd (10.9, 1.0)	14-OH 12
14-OH	2.71	d (10.9)	14
15	1.19	d (1.0)	1 ax 5
16	4.74	dd (2.0, 1.5)	5 2
18	2.25	sep (7.1)	19 20
19	1.02	d (7.1)	18 20
20	0.90	d (7.1)	18 19

nOe difference experiments

Proton irrad.	1 ax	1 eq	2	2 OH	5	6 ax	6 eq	7	11	12	14 OH	14	15	16	18	19	20
1ax/ 15		s	m		m				s				m				
1eq/ 6ax	m		s		m		s	w	m				s	w			
2	w	m											m				
5	m																
11	m	w								s							

s = strong, m = medium, w = weak

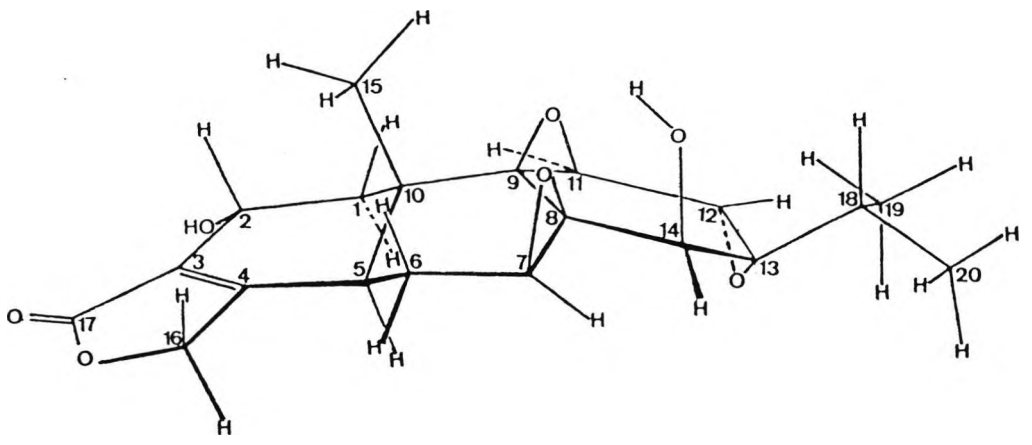


Table 5-16 : 1H NMR and nOe experiments on fraction P8 (Tripterolide)

Table 5-17: ^{13}C Data ($CDCl_3$) TRIPTEROLIDE (P8)

Carbon	Chemical shift (ppm)		
1	28.25		
2	61.47		
3	161.32		
4	127.24		
5	40.80		
6	23.40		
7, 11, 12*	59, 85	56.93	54.48
8, 9, 13*	66.37	65.74	60.69
10	35.81		
14	73.49		
15	14.75		
16	69.79		
17	Not seen		
18	38.82		
19	17.72		
20	16.88		

* Assigments uncertain within groups of signals.

5.5.5 Bioassay of subfractions

The results from the antifertility assays on rats are tabulated in Table 5-18 and Table 5-9 (p. 238)

Table 5-18
The effects of 402 series on the fertility of male rats.+

Extract code (no. rats tested)	Body wt gain (g)	Weight of testis (g/Kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml	%w/w starting material	
Control(5)	106±33	1.51±0.07	83±3	90±4		
442-P7(5)	97±23	1.65±0.16	82±3	82±7	42.75	
442-P8(5)	94±54	1.59±0.08	78±5	85±6	3.75	
442-P9(5)	99±52	1.53±0.19	78±4	87±5	2.22	
442-P10(5)	96±25	1.63±0.09	76±6	86±5	0.41	
442-P11(5)	117±11	1.64±0.15	78±6	86±5	0.1	
Control(5)	90±11	1.51±0.07	82±4	83±5		
442-P12(5)	87±18	1.50±0.11	0.1±0.2**	27±8**	1.42	A
442-P13(5)	92±13	1.55±0.11	78±3	69±7	2.94	
442-P14(5)	92±9	1.46±0.07	80±4	73±3	1.41	
442-P15(5)	109±19	1.67±0.07	80±4	72±4	2.53	
442-P16(5)	105±9	1.57±0.12	28±30*	47±13*	2.53	A
442-P17(5)	98±20	1.38±0.08	81±4	72±6	3.14	
442-P18(5)	98±17	1.52±0.08	77±5	70±6	7.6	
Control(5)	113±24	1.5±0.09	87±4	87±5		
442-P19(5)	123±10	1.64±0.05	85±3	97±7	9.83	
442-P20(5)	135±17	1.59±0.08	8026	80±4	3.85	
442-P21(5)	131±26	1.63±0.12	82±5	82±6	5.45	
422-P22(5)	114±27	1.61±0.23	83±4	85±6	1.42	
422-P23(5)	121±14	1.61±0.12	83±5	87±6	2.84	
422-P24(5)	116±28	1.56±0.11	81±6	84±7	2.94	
422-P25(5)	111±31	1.55±0.08	83±8	89±6	2.84	

+ Data expressed in x±SD, if applicable
A Active fraction
*P<0.01, **P<0.001, in comparison with the controls.

Overall, the results shown in Table 5-18 lead to the following conclusions:

1. Bioassay results confirmed that all of the activity was located in the 5th of the 6 fractions (P1 - P6) into which the eluate had been divided and analytical HPLC confirmed that this corresponds to the same group of peaks as found in fractions 422-16 to 422-19 obtained in the earlier work. 19 subfractions were generated by the subdivision of P5 and coded P7 - P25.

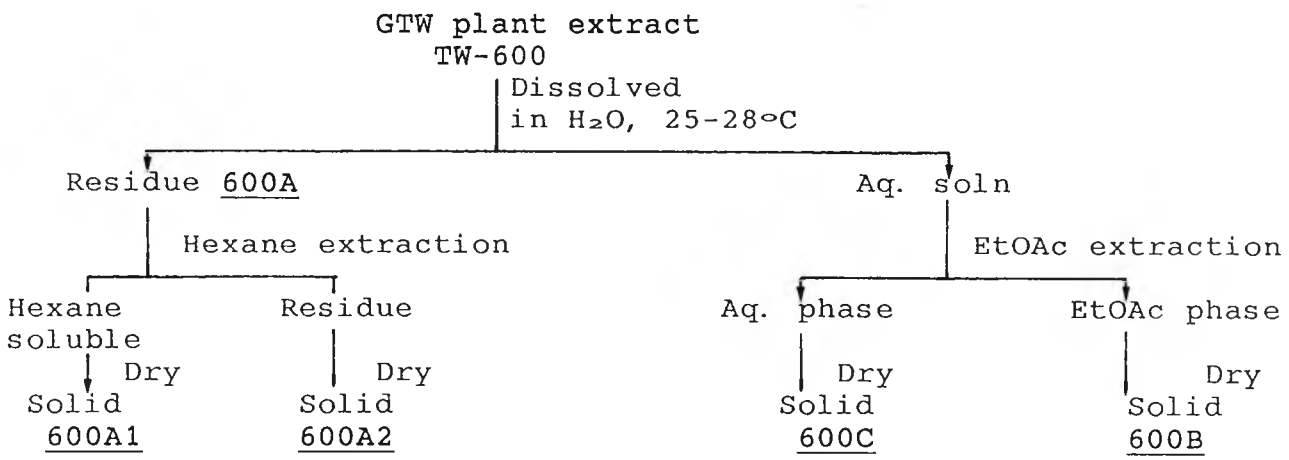
2. Fraction P12, which has been identified as tripdiolide, is the main antifertility component responsible for the activity seen in the aqueous phase and previously located in the multicomponent subfraction P5.

   It will be noted that a total dose of 1.4 mg was used in the bioassay of this fraction: on division into 30 daily doses for 5 rats in the test group, this means that the dose showing activity is only 1400/150 = 9.3 µg/rat/day.

3. Fraction P16 (triptolidenol, structure 11) is moderately active at the tested dose level of 2.5 mg total dose. The presence of activity in this compound is not at all surprising in view of its close structural relationship to triptolide and tripdiolide. It may be conjectured that other, related structures such as P8 and P13 will also be found to be active when they are tested at a sufficiently high dose level. The variation in levels of activity compared with the isomeric tripdiolide is of great importance in signifying the potential for optimization of activity/toxicity by studying further isomers and analogues.

5.6 Studies of Series 600 powders

A sample of 50 g of powdered plant extract was obtained, which was initially understood to be equivalent to the material used in tablet manufacture. However, it has always been understood from Chinese sources that the TW tablets contain a water soluble fraction which is termed "glycosidic" or "multiglycosides". On work-up of Series 600 material using our standard protocol for tablets (Scheme 5-3), it quickly became apparent that this material did not conform to such a description: on mixing with water, 98.5% of the mass was recovered as insoluble (600A) and only 1.5% as water-soluble. Moreover, almost all of the material which dissolved in water could be extracted from the aqueous solution with ethyl acetate (600B): thus, only 0.05% of the Series 600 material formed an aqueous residue (600C) which could be considered to be equivalent to the aqueous residue (e.g. 402) typical of tablet extracts. A sample of the water-insoluble material (600A) was extracted with hexane, which removed only a small amount of non-polar solubles (600A1), most being insoluble (600A2).



Scheme 5-3: Extraction sequence for TW-600 series

Table 5-19
The effects of 600 series on the fertility of male rats.+

Group(n)	Body wt gain (g)	Weight of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6	%w/w starting material	
Control(5)	91±26	1.57±0.12	85±4	108±6		
600A (5)	77±38	0.68±0.08	0.4±0.9	22±2	100	A
600A1(5)	82±23	1.55±0.15	81±2	86±9	0.07	
600A2(5)	80±21	0.55±0.08	2.6±4.2	18±3	98.4	A
600B(5)	97±12	1.35±0.05	82±6	88±8	1.5	
600C(5)	86±25	1.41±0.09	82±3	97±5	0.05	

+ Data expressed in x±SD, if applicable
A Active fraction

In view of this disparity, it was considered to be important to investigate the potency of the material as a preliminary to subfractionation. A series of samples of 600 powder of different weights were therefore submitted to Nanjing. On the basis that "1 test equivalent" (i.e. 180 tablets) of TW tablets would contain 180 x 10 mg = 1.8 g TW extract, doses were chosen as indicated (Table 5-20).

Table 5-20
The effects of 600 series on the fertility of male rats.+

Group(n)	Body wt gain (g)	Weight of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml	Test equi-valent	Sample sent (mg)	
Control(5)	82±19	1.67±0.14	88±3	92±4			
600-L1(5)	65±0.10	1.62±0.08	0	28±6	0.5	901	A
600-L2(5)	82±0.11	1.42±0.09	0.2±0.3	65±10	0.1	181	A
600-L3(5)	83±10	1.44±0.10	1.1±1.3	49±4	0.05	90	A
600-L4(5)	80±18	1.44±0.11	90±4	97±2	0.01	18	
600-L5(5)	70±22	1.46±0.08	85±5	95±5	0.005	9	

+ Data expressed in x±SD, if applicable
A Active fraction

The results shown in Table 5-20 indicate that the antifertility potency is present in this powder at the L3 level of dilution, i.e. 90 mg for 30 x 5 rat-day doses: the individual dose will have been 0.6 mg/rat/day.

Following the extraction methods discussed above (Scheme 5-3), the subfractions 600A - 600C were assayed at the level of "1 test equivalent" - i.e. material derived from 1.8 g powder, which is 20 times the activity limit of the crude mixture. The results (Table 5-19) show that all the activity resides in the water-insoluble portion (600A) which constitutes the bulk of the material. The fraction 600A2 and even the crude material 600 could be considered to be equivalent to the ethyl acetate fraction (e.g. 405) typical of tablet extracts. As explained previously, the three early subfractions, 435-01-1 to 435-01-3, of the ethyl acetate extract of the Series 400 tablets were accidentally lost, hence it was hoped to study these 3 subfractions of the material from Series 600, while also isolating triptolide.

5.6.1 TLC silica

A sample of 600A2 was then subjected to preparative TLC (SiO_2, 100% EtOAc instead of the previously used 90:10 $CHCl_3$/MeOH) and six fractions (T1 - T6) collected. Most of the material (80%) was collected in an orange band (600A2-T3) and bioassay (Table 5-21) showed that this contained all the activity.

Table 5-21
The effects of 600 series on the fertility of male rats.+

Group(n)	Body wt gain (g)	Weight of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6	%w/w starting material	
Control(5)	80±4	1.55±0.03	82±3	99±8		
600-T1(5)	62±12	1.50±0.13	75±6	100±9	4.19	
600-T2(5)	65±14	1.49±0.08	76±4	87±7	9.87	
600-T3(5)	55±11	0.51±0.09	0	13±6	73.64	A
600-T4(5)	61±10	1.42±0.13	67±6	95±10	8.97	
600-T5(5)	88±18	1.53±0.23	84±4	86±6	2.33	
600-T6(5)	111±25	1.54±0.08	75±4	120±28	1.04	

+ Data expressed in x±SD, if applicable
A Active fraction

5.6.2 Silica column chromatography

It was hoped that a faster processing time could be achieved on classical preparative column chromatography as compared to preparative TLC-silica, without sacrificing too much resolution. The columns were slurry packed with 40-60 μm silica in chloroform. The eluent used was 90:10 $CHCl_3$/MeOH and the sample was dissolved in chloroform. The collected fractions could be monitored for the presence of tripdiolide, triptolide or triptolide chlorohydrin by analytical TLC, as described earlier (Chapter 5.4.2). It was found that when 500 mg of 600A2 was loaded onto 200 g of silica packing, an enriched fraction was collected, which accounted for 65% of the starting material. This amount increased to 90% recovery when up to 2 g of 600A2 was loaded on 200 g of silica.

When the enriched fraction was compared on analytical HPLC with the starting material, no difference could be observed on analytical HPLC between the chromatographed material and the starting material. Taking into account also that not too much inactive material had been removed, this technique was abandoned. The only advantage that could be seen from this chromatography was that the most polar material was left behind, adsorbed on the silica, avoiding the irreversible adsorption on the HPLC packing material on further treatment. In view of the lack of selectivity of the preparative TLC and column chromatography separation, it was concluded that preparative HPLC would be necessary for efficient separation.

5.6.3 HPLC: ODS continuous gradient elution

A sample of 600A2 was initially submitted to a continuous gradient elution, using the new 1" O.D. Vydac-ODS 15-20 μm column, protected with a pre-column containing the same packing material. The idea was to use this larger particle size and more economic material to achieve the initial subfractionation into polarity groups. Then the active subfraction could be further chromatographed using the expensive, high performance preparative ODS-Exsil 5 um material, ideally under optimum isocratic conditions to obtain pure components. This ODS-Exsil 5 μm was also protected with the previously used Vydac-ODS precolumn, to avoid contaminating the expensive preparative column. A test 100 mg sample of 600A2 was separated by preparative gradient chromatography on the Vydac ODS column into 6 fractions (Figure 5-40): The initial subfractions 600-F1 to 600-F3 should correspond to 435-01-1 to 435-01-3, respectively. As anticipated, the major single component of 600-F3 was tripdiolide, as seen on HPLC analysis using the H_2O/THF system developed for the resolution of tripdiolide (Section 5.5.3). No tripdiolide or other compounds containing the same chromophore were seen either in 600-F1 or 600-F2. Fraction 600-F5 contains triptolide and corresponds to 435-08, although 600-F5 is much more impure as the HPLC analysis in Figure 5-41 indicates.

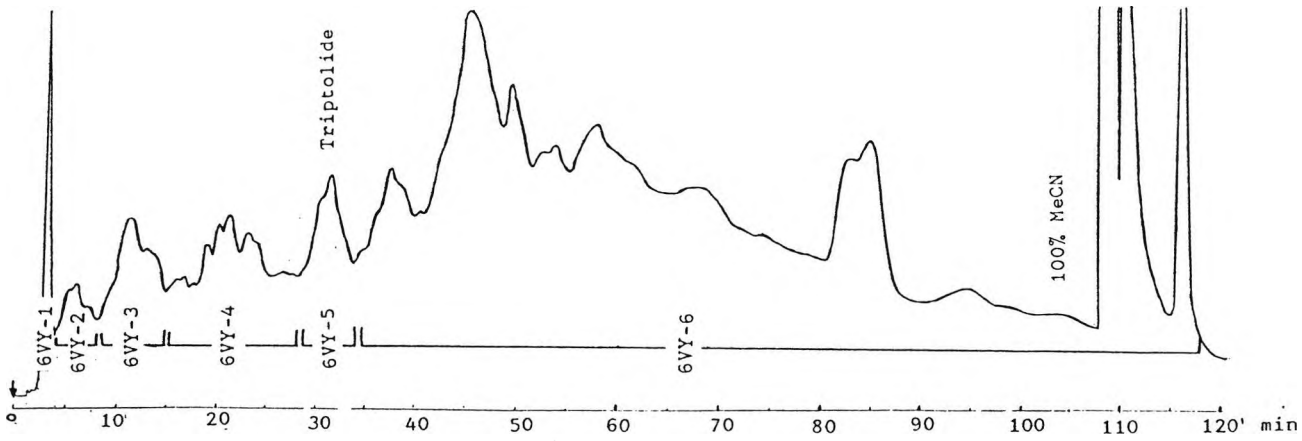


Figure 5-40. Preparative HPLC separation of 600A2 to obtain the active fraction 6VY-3 (contains tripdiolide) and 6VY-5 (contains triptolide). 100 mg sample was injected using a 1 ml loop on a 1" O.D. x 50 cm preparative Vydac-ODS 300 Å 15-20 µm column, and eluted with continuous gradient from 10:10:80 to 22:22:56 MeCN/MeOH/H_2O at 30 ml/min using a 1 l mixing flask, monitored at 230 nm and 0.02 AUFS using an analytical flowcell.

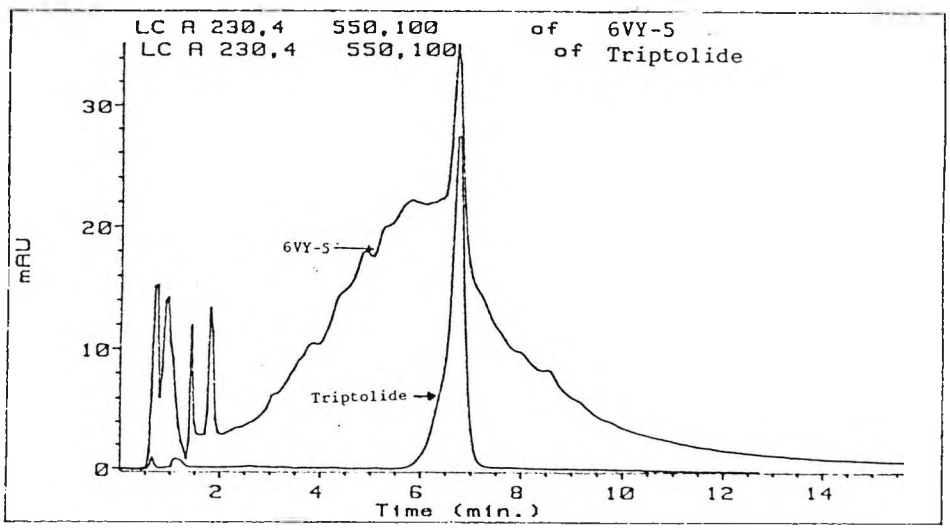


Figure 5-41. Superimposed chromatograms of the preparative subfraction 6VY-5 and a reference sample of triptolide, run under isocratic conditions on a 25 x 0.45 cm Hypersil-ODS 5 µm column, eluted with 28:72 MeCN/H_2O at 2 ml/min, monitored at 230 nm.

5.6.4 Nitro-Nucleosil HPLC

Series 600 was also studied on the Nitro-Nucleosil material for the resolution and separation of tripdiolide and any other related isomers; this was done mainly to evaluate the more general application of this expensive phase to justify its acquisition. Bad resolution was obtained on the crude TW 600; small changes in the proportion of modifier in the mobile phase did not improve resolution considerably. Under the chromatographic conditions used in Figure 5-42, triptolide, triptolide chlorohydrin and tripdiolide eluted in this order, all of them unresolved from the other components of the crude series 600 extract (Figure 5-42). Clearly the limited application of the use of this stationary phase just for the resolution of highly purified subfractions, would not justify the investment and effort necessary to prepare a preparative HPLC column containing this phase.

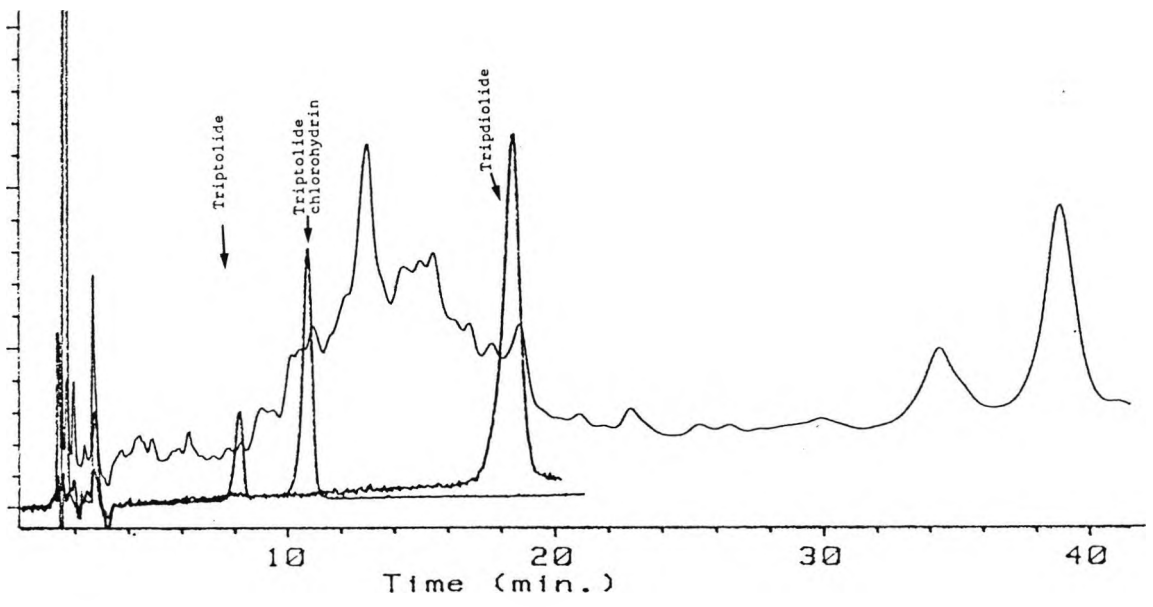


Figure 5-42. Normal phase HPLC separation of the crude fraction TW600 on 25 x 0.45 cm Nucleosil-nitro bonded 5 µm column, eluted with 80:5:5:10 Hexane/CH_2Cl_2/THF/MeOH at 2 ml/min, monitored at 230 nm. Comparison with the active reference samples of triptolide, triptolide chlorohydrin and tripdiolide.

5.6.5 600-F3: ODS silica HPLC

It was therefore decided to use the THF/H_2O mobile phase on the silica-ODS column for the subfraction of 600-F3.

A 4/96 THF/H_2O mobile phase was used on a 1" O.D. preparative Exsil-ODS 5 µm column, as in Figure 5-43. The optimum preparative HPLC conditions were developed and were taken over by another co-worker in the laboratory, who will process large amounts of material to provide WHO collaborators with compounds for detailed biological investigations.

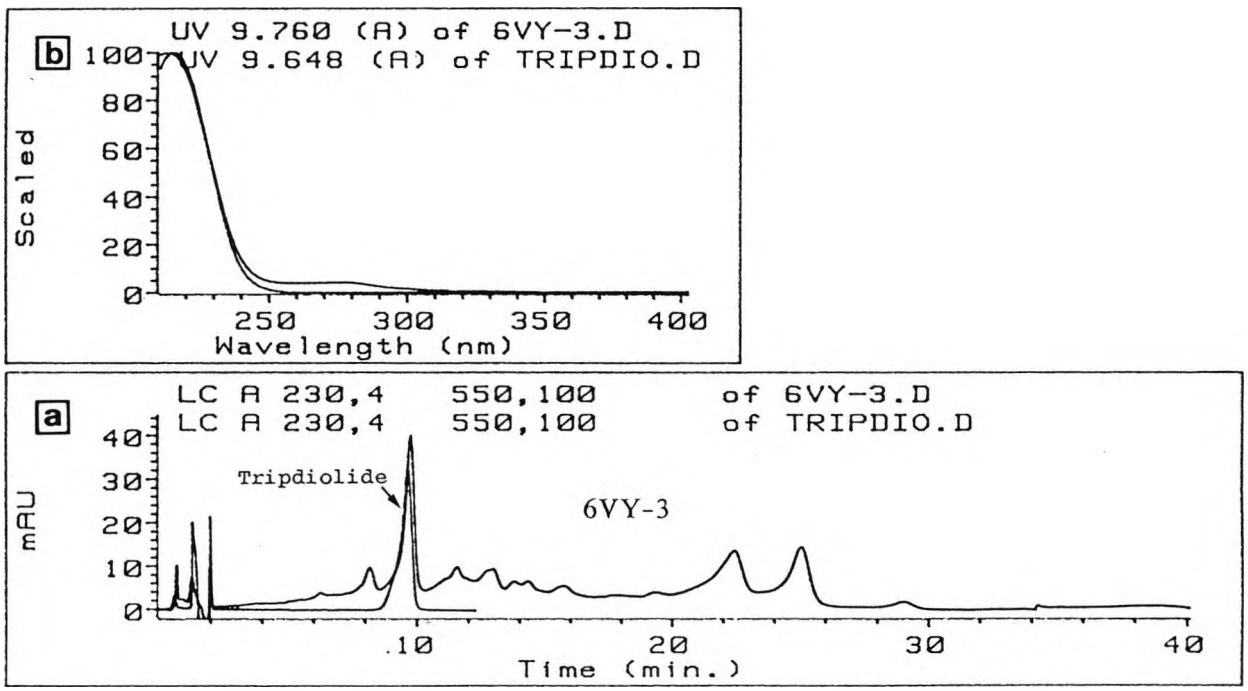


Figure 5-43. a) Superimposed analytical chromatograms of the preparative subfraction 6VY-3 and a reference sample of tripdiolide; run on a 25 x 0.45 cm Exsil-ODS 5 µm column with 96:4 H_2O/THF at 2 ml/min and monitored at 230 nm with a HP 1040 photodiode array detector. b) Superimposed UV spectra of the main peak of 6VY-3 and the reference sample of tripdiolide. The main component in 6VY-3 is tripdiolide.

5.6.6 HPLC silica chromatography: resolution of triptolide

A sample of TW extract, from which had been removed the more polar components by initial silica column chromatography or reverse phase silica-ODS HPLC, could be injected on a HPLC silica column, without fear of blocking the top of the column. A chromatographic system of 99:1 CH_2Cl_2/MeOH (Figure 5-44) was found to elute triptolide early and reasonably resolved, resembling the analytical high performance silica TLC results described earlier (Chapter 5.4.2).

It would be very convenient to transfer the chromatographic conditions of this analytical system to a preparative scale HPLC system to isolate triptolide from enriched fractions quickly and easily and with the added advantage of the use of low-boiling solvents.

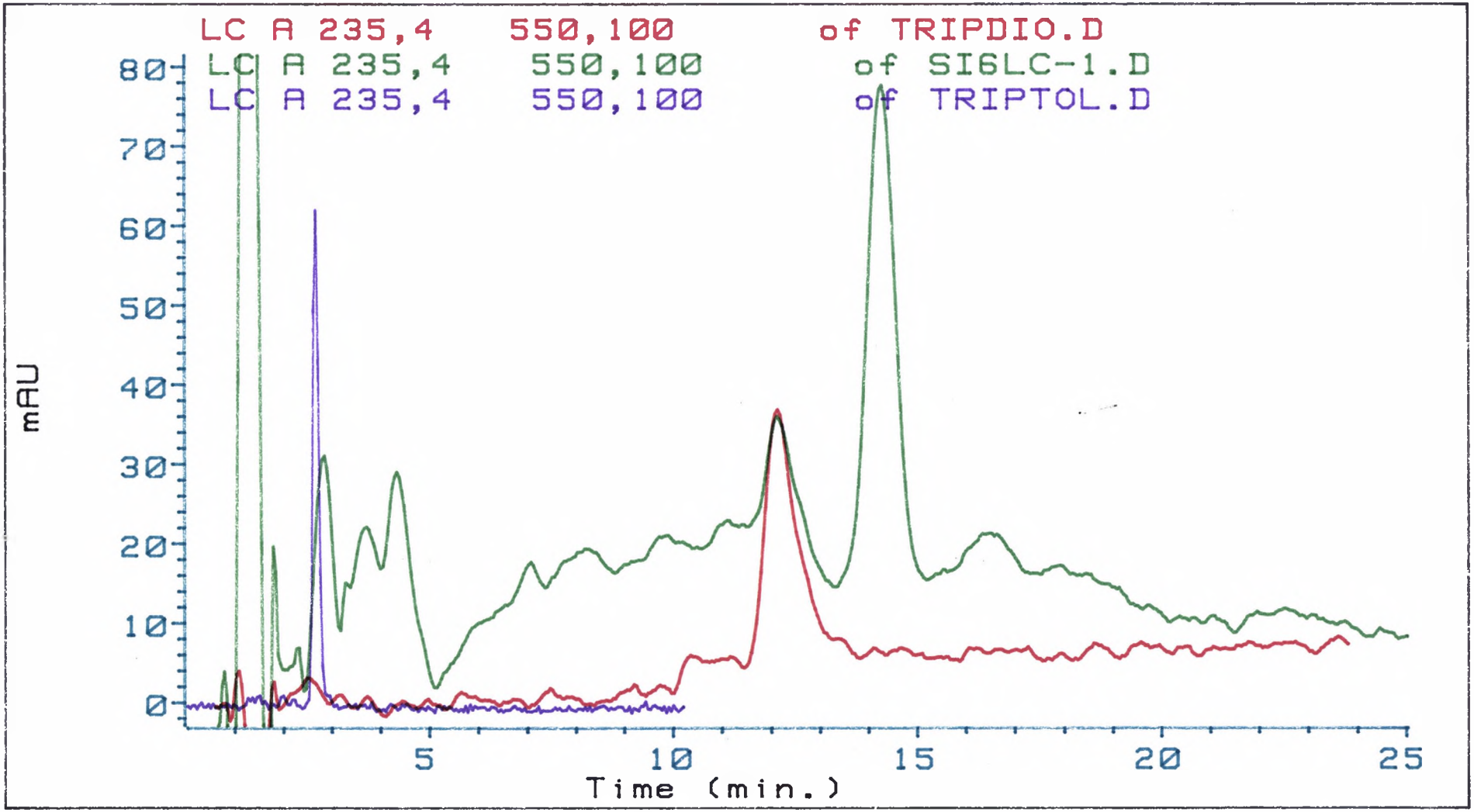
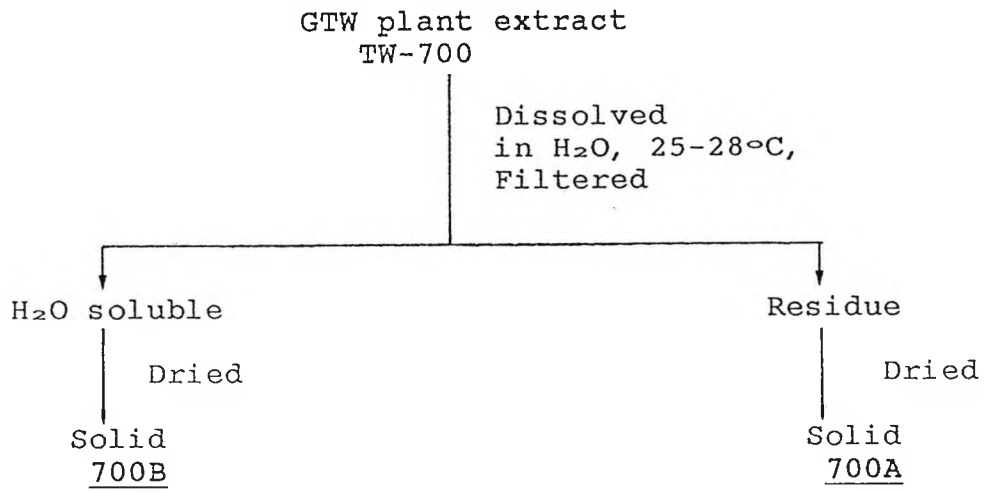


Figure 5-44. Normal phase HPLC method on Hypersil-silica to resolve the active materials triptolide and tripdiolide from the crude extract TW600. Samples were run on a 25 x 0.45 cm Hypersil-silica column, eluted with 99:1 CH_2Cl_2/MeOH at 2 ml/min and monitored at 230 nm.

5.7 Studies of Series 700

Concurrent with the work on Series 600, we have similarly shown that most of the Series 700 powder (Scheme 5-4) is insoluble (700A) rather than water soluble (700B) and that the activity resides in the insoluble part (Table 5-22). A dose-response study, using identical weights to those selected in the 600 Series above, showed that the 700 material is less active (Table 5-23). Thus, only at the L1 level (900 mg tested: 0.5 test equivalents) was there a significant effect on epididymal spermatozoa.



Scheme 5-4: Extraction sequence of TW-700 series

Table 5-22
The effects of 700 series on the fertility of male rats.+

Group(n)	Body wt gain (g)	Weight of testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6	%w/w starting material
Control(5)	109±19	1.55±0.08	88±3	115±2	
700A(5)	85±14	1.27±0.08*	2.8±2**	8.8±0.6	99.8 A
700B(5)	103±22	1.67±0.21	81±2	122±6	0.2

+ Data expressed in x±SD, if applicable
A Active fraction
* $p<0.01$, ** $p<0.001$, in comparison with the control.

Table 5-23
The effects of 700 series on the fertility of male rats. +

Group(n)	Body wt gain (g)	Weight testis (g/kg)	Epididymal sperm % live sperm	Epididymal sperm Density 10^6/ml	Test equi-valent	Sample sent (mg)	
Control(5)	85±33	1.85±0.20	88±3	87±3			
700-L1(5)	96±24	1.41±0.49	0	23±13	0.5	900	A
700-L2(5)	80±18	1.39±0.21	68±18	80±10	0.1	180	
700-L3(5)	85±11	1.31±0.33	66±20	73±21	0.05	90	
700-L4(5)	83±18	1.40±0.19	76±14	82±13	0.01	18	
700-L5(5)	90±28	1.59±0.15	72±3	87±6	0.005	10	

+ Data expressed in x±SD, if applicable
A Active fraction

5.8 Conclusion

My investigation of the TW tablets and powders obtained from China has revealed several compounds which are active as male antifertility agents as measured by the marked decrease in sperm motility and sperm density in treated rats. All the active compounds have the diterpene triepoxide structure and they have been characterized as : triptolide, tripdiolide and triptolidenol. An additional active component is triptolide chlorohydrin, which is an artifact found in some TW tablet batches and powders. It is speculated that this chlorohydrin derivative of triptolide was probably formed during the extraction of the roots of Tripterygium wilfordii, involving some hydrochloric acid treatment. This hypothesis was confirmed by transforming pure triptolide to its hydrochloride derivative, when it was reacted with hydrochloric acid gas dissolved in acetonitrile. It was surprising that the opening of the epoxide link had occurred in the 12,13 position; this was different from Kupchan's theory about the mechanism of action of triptolide as an antileukemic agent. Kupchan postulated that an intramolecular catalysis by the 14-hydroxyl group may assist in the selective nucleophilic attack of biological macromolecules on the 9,11-epoxide. The opening of the 12,13-epoxide by acid protonation and nucleophilic attack of the chlorine ion could be going through a different mechanism to that involved in thiol attack. It has been observed that this triptolide chlorohydrin reverted to the initial triptolide, just by standing in solvents such as water, methanol, acetonitrile or chloroform.

During the isolation of active compounds, all the diterpene triepoxides found in TW were separated in a pure form, and their structural elucidation was assisted by NMR and MS spectroscopy. These diterpene triepoxides were identified as: triptolide, tripdiolide, triptolidenol, tripterolide and 19-hydroxytriptolide, from which only triptolide and tripdiolide were fully active, while triptolidenol was partially active at the dose tested. Tripterolide and 19-hydroxytriptolide were non-active at the calculated 5 test equivalent dose. It is possible that these two isomers of triptolide are not contained in sufficient quantity to be tested above their threshold activity level, and a dose response study be recommended on all the isolated diterpene triepoxides; this study would correlate the activity found in the diterpene triepoxides with the small modification in the position of the hydroxyl groups of the tripdiolide isomers.

Triptolidenol[6] and tripterolide[7] had been previously isolated and identified by Chinese scientists during phytochemical studies. No literature reference has been found to the isolation or identification of 19-hydroxytriptolide, and therefore we believe we have isolated and identified a new diterpene triepoxide from Tripterygium wilfordii.

A protocol to isolate all the diterpene triepoxides from the TW extract could be proposed based on the knowledge gained during this project. Initially, two enriched fractions, one containing the polar diterpene triepoxides and the other containing just triptolide, could be separated while simultaneously removing easily recognized non-active fractions; this could be accomplished by running a continuous water/methanol/acetonitrile gradient on a silica-ODS column.

Tripdiolide, and the three other isomers tripterolide (P8), triptolidenol (P16) and 19-hydroxytriptolide (P13) could be then isolated as nearly pure components by chromatographically separating the polar diterpene triepoxide fraction on a silica-ODS HPLC column with an isocratic THF/water mobile phase system. A nearly pure sample of triptolide could be then isolated from the enriched fraction by eluting it from a silica column with a isocratic dichloromethane mobile phase slightly modified with methanol. This last chromatographic system has been developed on analytical HPLC but it has not yet been tested on a preparative scale. As the very polar components of the crude TW will have been previously removed from this triptolide enriched fraction, blockage of the top of the silica column is not anticipated.

At a WHO Steering Committee meeting in November 1989, interest was shown in the isolated and identified diterpene triepoxide from the TW extract. A Use Patent is being prepared for filing at the moment for the use of these diterpene triepoxides as potential oral male antifertility agents.

5-7 References

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