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Novel Derivatives of Sporopollenin for Potential Applications in Solid Phase Organic Synthesis and Drug Delivery

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ABSTRACT

The outer coat (exine) of plant pollen grains and spores is composed of the material sporopollenin. Sporopollenin is exceptionally resistant to chemical and physical attack and is perhaps one of the most chemically resistant, naturally occurring organic materials known. Its stability to chemical and physical stress is illustrated by its survival in ancient sedimentary rock of around 3.7 billion years old.

Sporopollenin from *Lycopodium clavatum* has been functionalised in the past for use in the preparation of peptides and for use in ion and ligand exchange. It was found that sporopollenin had a number of advantages over the synthetic resins commonly used in solid phase synthesis which included a constant chemical structure, constant pore size, chemical and physical stability and commercial availability.

Investigations into the isolation of sporopollenin using various procedures outlined in the literature were reviewed in order to find the most efficient protocol for sporopollenin preparation. Detailed studies of the sporopollenin were undertaken to establish its chemical and physical stability, swelling, filtering and hydration characteristics. Results indicated that sporopollenin would make a good alternative to other solid supports as it showed little swelling in organic solvents, was easily filtered through grade 3-4 sinters and the size of the sporopollenin was constant. Also, it could be dried efficiently and showed good chemical and physical stability, e.g. it can be refluxed for several days in strong acid and base and a variety of organic solvents without decomposition. It also can be stirred for several days with little degradation. In contrast, a parallel study with a commercial PS-DVB resin showed it to be considerably less stable, e.g. stirring for one day resulted in severe degradation of the

resin. Also, most of the commercially available resins are found to have a variable particle size and variable loadings.

An investigation into the types of functional groups present on the sporopollenin surface was undertaken to explain the types of functionalities present on sporopollenin and examine their reactivity. The objective was to assess the potential of sporopollenin for use as a solid phase support for synthesis and drug delivery. Studies indicated that ketone, acid and hydroxyl groups were present. Thus, in order to obtain uniformity of functionality sporopollenin was reduced with the objective of obtaining solely hydroxyl groups on its surface. Suprisingly the loading of hydroxyl groups varied between one batch to another (0.40-0.92 mmol/g). Attempts to halogenate the hydroxy groups with PCl_5 and $SOCl_2$ or condense them with amino acids to form ester linkages was only moderately successful. In contrast, chloromethylation of sporopollenin using dimethoxymethane and $SOCl_2$ with $ZnCl_2$ as a catalyst gave consistent levels of chloride loading from one batch to another (1.01-1.28 mmol/g). Such a form of chloromethylated sporopollenin could be compared to Merrifield resin. Preliminary attempts to substitute the chloride proved difficult due presumably to the attachment of chloromethyl groups to aliphatic sites rather than aromatic sites. Amination of different batches of sporopollenin with primary amines, e.g. n-butylamine gave loadings of around 1-2 mmol/g. An extensive study was undertaken to assess the stability of the amine attachment to acid, bases and organic solvents. In all of these a relatively robust attachment was found with no less than 70% of the amine still attached. This study encourages the use of diamines as linker groups with the intention that one primary amine would attach to the sporopollenin with the other being available for further substitution to attach either synthons or drugs.

ABBREVIATIONS

Ala	Alanine
Boc	t-Butoxycarbonyl
CHA	Cyclohexylamine
COSY	Correlation spectroscopy
DCC	*N, N'*-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DEAD	Diethyl azodicarboxylate
DIBAL-H	Diisobutylaluminium hydride
DIPEA	Diisoproptlethylamine
DMA	*N, N*-Dimethylacetamide
DMAP	4-Dimethylaminopyridine
DMF	*N, N*-Dimethylformamide
DNPH	2,4-Dinitrophenylhydrazine
Fmoc-Cl	9-Fluorenylmethyl chloroformate
GC	Gas chromatography
HMP	4-Hydroxymethylphenoxy
HOBt	*N*-Hydroxybenzotriazole
IR	Infrared
MMNO	Methylmorpholine-*N*-Oxide
NMR	Nuclear magnetic resonance
PEG	Poly(ethylene glycol)
PEGA	Poly(acrylamidopropyl-PEG-*N,N*-dimethylacrylamide)
Phe	Phenylalanine

PM	Portioning-mixing
Pro	Proline
PS	Polystyrene
PSA	Preformed symmetrical anhydride
PyBop®	Bentotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate
SEM	Scanning electron microscope
SPOS	Solid phase organic synthesis
TEA	Triethylamine
TFA	Trifluoroacetic acid
TFMSA	Trifluoromethanesulfonic acid
THF	Tetrahydrofuran
UV-Vis	Ultraviolet-visible
Val	Valine
XPS	X-ray photoelectron spectroscopy

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1 THE USE OF INSOLUBLE SUPPORTS IN ORGANIC SYNTHESIS

1.1 INTRODUCTION

Insoluble supports are used in organic synthesis in a number of ways which include:

a) Solid phase organic synthesis (SPOS).[1-7]

b) The use of immobilised agents such as scavengers, reagents and catalysts to aid solution phase chemistry.[1,2,8-13]

Solid phase organic synthesis (SPOS) is a process where molecules are synthesized whilst attached to an insoluble support. Solid phase synthesis has been used for nearly 40 years and the concept was first developed in 1963 by Merrifield[14] for the synthesis of peptides and independently by Letsinger.[15] The solid phase approach to the preparation of peptides, introduced by Merrifield,[14] involved the attachment of an *N*-protected amino acid covalently to a functionalised polystyrene (PS) support cross-linked with 2% divinylbenzene (DVB) in the form of spherical beads. *N*-Protected amino acids were then coupled one at a time and remained bound to the support throughout the synthesis. After synthesis, the peptide was then conveniently purified by filtration and washing of the solid support to remove excess reagents and any by-products. The final peptide was then cleaved from the support. Merrifield found that this procedure simplified and shortened peptide synthesis considerably.

This solid phase method has also been developed and used for oligonucleotide[16] and oligosaccharide synthesis.[17,18] In the 1970's it was realised that insoluble polymers could be used for the synthesis of organic molecules and a number of papers reviewing the

early organic syntheses on solid supports were published.[10,19,20] One early application of SPOS was the preparation of insect sex attractants by Leznoff and co-workers in 1976.[21] Solid phase organic synthesis never really became popular in organic synthesis until the last decade when an explosion of interest occurred. To date large numbers of publications and reviews in SPOS have been produced which show the wide range of reactions which can be carried out on the solid phase and demonstrates the popularity and versatility of this method.[1-3,5,8,22-25]

Solid supported reagents, scavengers and catalysts have been used in organic chemistry since the 1960's.[1,2,9,-13,26]

Solid supported reagents are reactive organic groups attached to a solid support which transform a substrate into a new chemical product.[9,12,27] The substrates are in solution and the reagents are immobilised onto the support, at all times. By-products from the reaction remain bound to the support.

Solid supported scavengers are reactive organic groups which quench excess reagents and remove known impurities from solution phase reactions.

Solid supported catalysts are simply used in sub-stoichiometric amounts to effect transformation of a substrate.

These immobilised agents can be removed after the reaction by simple filtration. This area has received renewed attention and a wide range of these agents have been developed for useful applications in solution phase synthesis (See earlier references).

1.2 COMBINATORIAL CHEMISTRY

The main reason why solid phase organic synthesis and the use of solid supported agents have received renewed investigation is because of the development of a technique known

as combinatorial chemistry.[1,2,23,27-33,] This technique allows the simultaneous generation of large numbers of compounds quickly and efficiently. Combinatorial chemistry is becoming increasingly popular in research into new pharmaceuticals, agrochemicals and other materials such as superconductors and catalysts.[29,31,33-36]

Traditionally the development of drugs involves the identification of lead compounds that have some degree of activity for the desired biological target. These lead structures usually originated from the isolation and screening of natural products derived from plants, animal and microbiological fermentation sources. Once a potentially useful lead compound was found it was synthesized, purified and tested for biological activity. Optimisation of this lead structure was then carried out by synthesizing and evaluating many variations of the lead compound one at a time. Generally thousands of new compounds have to be prepared and screened in both lead compound identification and optimisation steps in order to find a potentially marketable product. The one by one synthesis of thousands of new compounds followed by their one by one testing made the drug discovery process time consuming, tedious and expensive.

More recently, automated screening methods which have the ability to test a large number of substances have been developed and introduced. This "robotic high-throughput screening" along with the advances in medical science, which has led to a greater understanding of disease processes, has increased the demand for more test substances to be prepared quickly and with reduced costs. The aim of the researchers is to speed up lead compound identification and lead optimisation steps by synthesising more compounds at a faster rate. This has led researchers to turn to combinatorial chemistry.

Combinatorial chemistry was originally developed for the preparation of peptide libraries[2,31,33] and has been efficiently adapted for the production of small organic molecules.[23,27,29,31-35] Combinatorial chemistry is a type of synthetic strategy which leads to the production of large numbers of related compounds. The collection of all the compounds in a combinatorial synthesis is known as a library.[23,32,34,36] These libraries can be prepared individually in parallel or as mixtures using solution phase or solid phase methods.[5,23,27,28,30,33]

1.2.1 Parallel Synthesis[2,23,27,30,31,33,36]

This approach is the most straightforward for the preparation of compound libraries. Products are synthesized in parallel in separate reaction vessels and are said to be spatially separate.[27] Chemists often use a 96 well microtitre plate for the preparation of compounds. An example of parallel synthesis is illustrated in Figure 1.1.[29] This involves the preparation of a series of amides form 8 different amines, (labelled A1 to A8) and 12 different carboxylic acids (labelled B1 to B12). In the first row of the microtitre plate, the A1 amine is added to each of the wells. In the second row the A2 amine is added to the wells and so on. These amines can be attached to the solid support or can remain in solution. Each carboxylic acid (B1 to B12) is then added to the appropriate well. The B1 acid is added to the first column, B2 to the second column and so on. From only 20 different building blocks, 96 different compounds can be made.

Early examples of parallel synthesis were developed by Geysen (1984)[37] and Houghten (1985).[38] Geysen[37] developed a multipin apparatus which allowed the multiple synthesis of 96 individual peptides in wells of a microtitre plate. Synthesis was carried out using polyethylene pins mounted in a supporting block and each pin fitted into a separate well

of the microtitre plate.[27,30] The pins were coated with a polymeric material which was further derivatised with polyacrylic acid to allow the attachment of the first amino acid. The pins were then placed in the wells which contained the dissolved amino acid and attachment took place. The peptide chain was built onto the pins through step by step coupling of the amino acids. After the reaction was complete, the peptides could be removed from the pins into the individual wells. This multipin procedure has been modified for the synthesis of organic libraries and one example is the preparation of a library of structurally diverse 1,4-benzodiazepines by Ellman and co-workers (1992).[39]

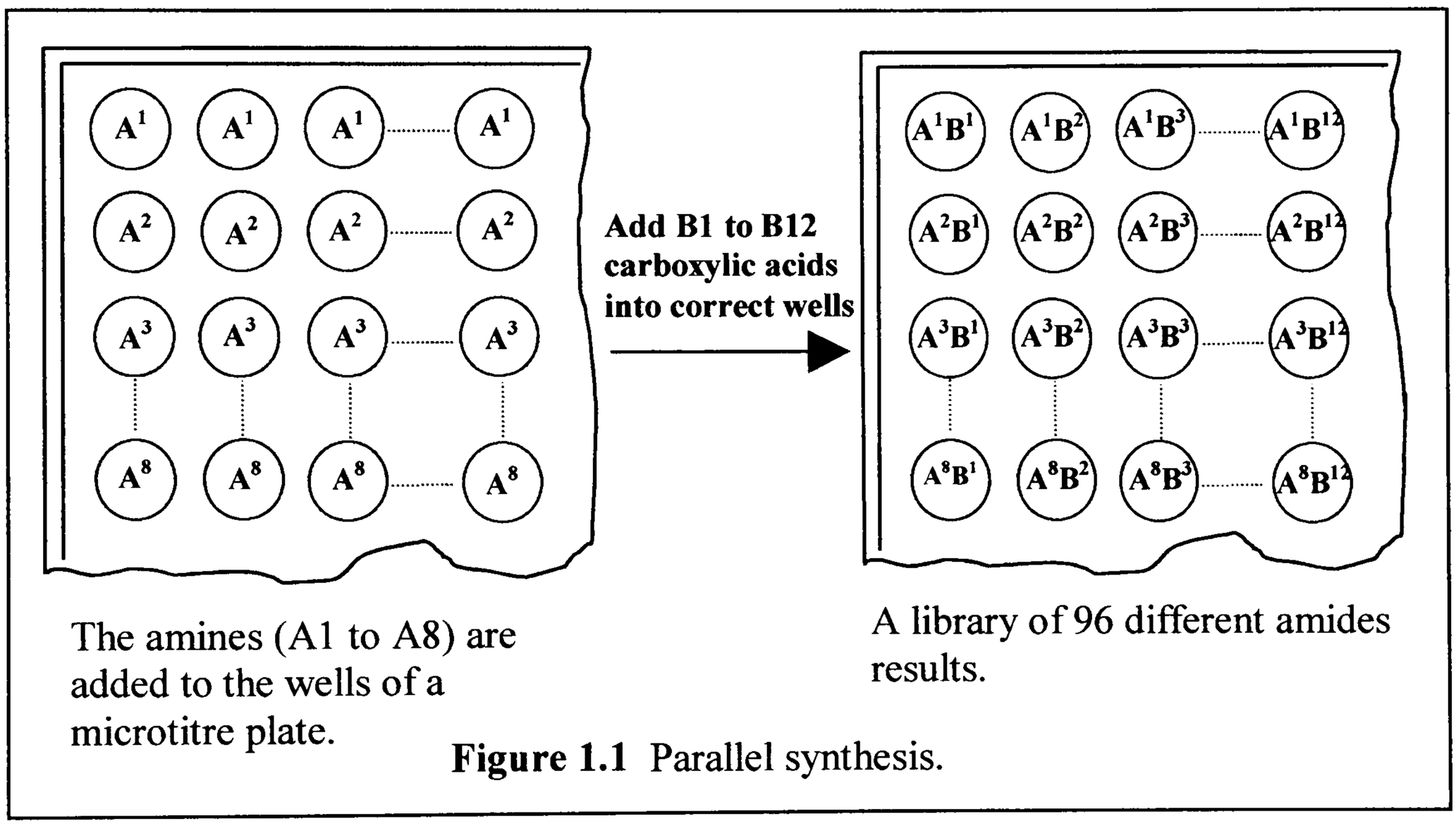


Figure 1.1 Parallel synthesis.

Houghten[38] introduced the tea bag method for multiple peptide synthesis. Resin beads were used which were contained inside porous polypropylene mesh bags. The resin beads could not escape but solvents and soluble reagents could enter the bags. The advantage of the tea bag method was that the bags could be placed in the same reaction vessel and procedures such as washing and removal of protecting groups could be carried out in one step.

1.2.2 Synthesis of Mixtures

The most popular method for the preparation of compound mixtures is the portioning-mixing (PM) method developed by Furka and co-workers.[40-42] This method was originally developed for the preparation of mixtures of peptides and was adapted for use by other researchers.[43,44] This method has also been used successfully for the preparation of large numbers of small organic compounds.[27,29,30,32,34,35] The PM method is usually carried out on solid resin beads. Firstly, the solid support is divided into equal portions and each portion is reacted with different reagents. The resin portions are then washed, combined, mixed thoroughly and then divided into the reaction vessels ready to perform the second synthetic step. By repeating the cycle of division, reaction and mixing, large compound libraries are produced with all the possible combinations of building blocks. An example of this procedure is given in Figure 1.2 where a 27 member library is prepared from just 9 building blocks.[23,27] The solid support is divided into three and each portion is placed in a separate reaction vessel and treated with a an excess of a different building block A, B or C. After the reaction and washing procedures the resin is then recombined, mixed and reapportioned into the three reaction vessels containing another set of different building blocks, D, E and F. This procedure is then repeated using building blocks G, H and I. At the end of the synthesis each bead ends up with a unique compound bound to it and all possible combinations of the trimers are produced. Large mixtures of compounds can be produced in this way. Once the compound mixtures have been prepared it is necessary to determine which substance in the mixture has the desired activity. There are a number of methods of doing this which include deconvolution by re-synthesis and screening processes[1,2,33,] and the addition of tags to every resin bead which keeps a track of the chemistry carried out on the

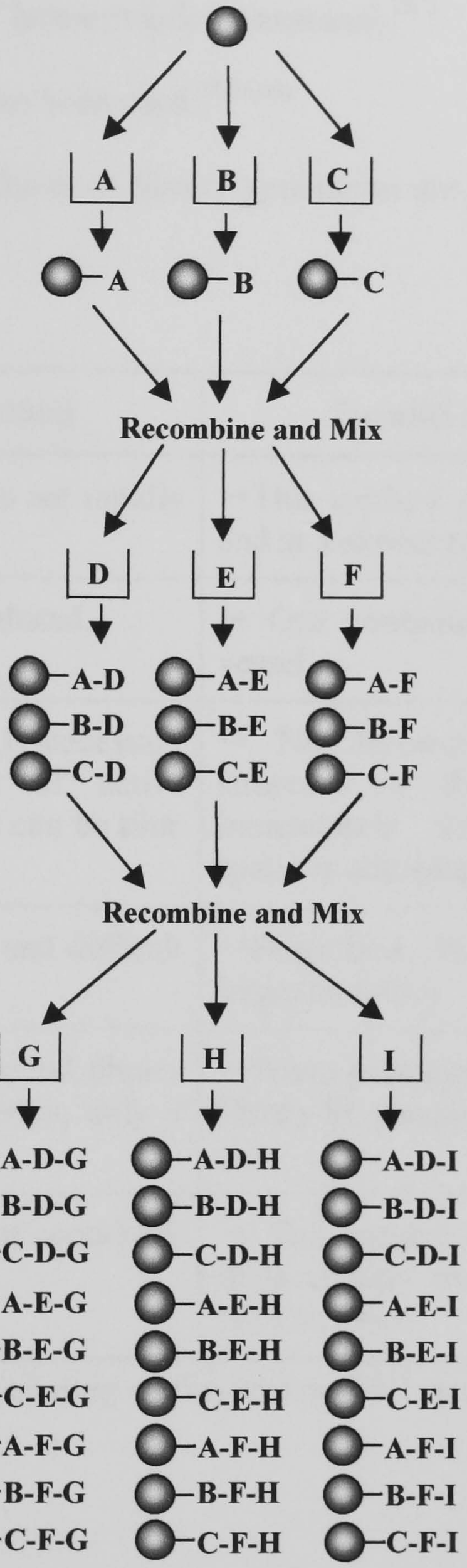


Figure 1.2 The preparation of a 27 compound library by using the portioning mixing method.

support. Various tagging systems have been developed such as chemical tagging with oligonucleotides, peptides and haloaromatic compounds.[28,33] Physical systems such as radiofrequency tagging have also been used.[28,33,45a]

A comparison of the two methods of library generation are summarised in Table 1.1 below.

Portioning Mixing Method	Parallel Synthesis Method
→ Large numbers of molecules are rapidly generated.	→This method generates fewer molecules and at a slower rate.
→ Mixtures of compounds produced.	→ One compound produced per reaction vessel.
→ Deconvolution or tagging is necessary to determine the structure of active compounds in a mixture. This can be time consuming.	→ No deconvolution necessary as the structure of the active compound is immediately known because of the spatially addressable format.
→ Follow up quantities costly and difficult to synthesise.	→Simplified follow up production of larger amounts.
→ When preparing a 27 compound library of trimers from 9 building blocks, only 9 reaction steps are necessary.	→When preparing the same 27 compound library 81 reaction steps need to be carried out.
→ A reduction in the number of containers required.	→ The number of containers required is large as only one compound is produced per container.

Table 1.1 Comparison of the portioning mixing and parallel synthesis method.

1.2.3 Automation

Numerous automated devices have been developed for the synthesis of organic compound libraries.[1,7,45b,46,47a] One of the first apparatus designed was the Diversomer™ apparatus[48,49] which is capable of running 40 reactions simultaneously. Numerous companies have launched commercial automated apparatus including Advanced

ChemTech,[1] Argonaut Technologies and[47] and Mettler-Toledo Myriad Ltd.[45b] These automated synthesisers are capable of solution or solid phase chemistry using parallel synthesis or portioning mixing methods. Examples of these are the BenchMark 440 Omega multiple organic synthesiser which is capable of solution and solid phase chemistry and can produce 40 discrete compounds or libraries. The BenchMark 384 high throughput synthesiser is capable of producing 384 different organic molecules or libraries.[1] Automation can result in the production of large numbers of compounds quickly and easily.

1.3 SOLID PHASE ORGANIC SYNTHESIS (SPOS)

1.3.1 Advantages of SPOS

Organic synthesis performed on the solid phase has a number of advantages over conventional solution phase synthesis which include:

a) Reactions can be driven to completion and high yields can be obtained using excess reagents.[1-4,7,14,23,25,27,33,50,51]

b) The intermediates and final products in the reaction sequence remain attached to the solid support at all times. This makes purification simple and rapid by filtration and washing to remove soluble by-products and excess reagents.[1-3,14,27,33]

c) Physical losses that are normally encountered during the isolation and purification of intermediates in solution by traditional methods such as crystallisation and chromatography are avoided.[2]

d) Toxic or hazardous substances can be attached to a solid support which reduces their toxicity enough to be handled safely with minimum risk to users and the environment.[2,26]

e) The solid phase process is highly suitable to automation and is well suited to the preparation of combinatorial libraries of compounds by PM or parallel synthesis methods (See Sections 1.2.1 and 1.2.2).

1.3.2 Solid Supports and Linkers

A number of different types of solid supports have been developed for solid phase synthesis. The most common supports used are usually synthetic cross-linked polymeric supports in the form of spherical beads which are typically 38 to 300 μm in diameter.[1,2,34] The most widely used and commercially available support is polystyrene beads cross-linked with 1-2% DVB.[1,2] This resin was first introduced by Merrifield in 1963.[14] Organic molecules are normally attached to the resin by a linker (See Figure 1.3). Linkers are considered to be insoluble, immobilizing protecting groups for solid phase synthesis.[3]

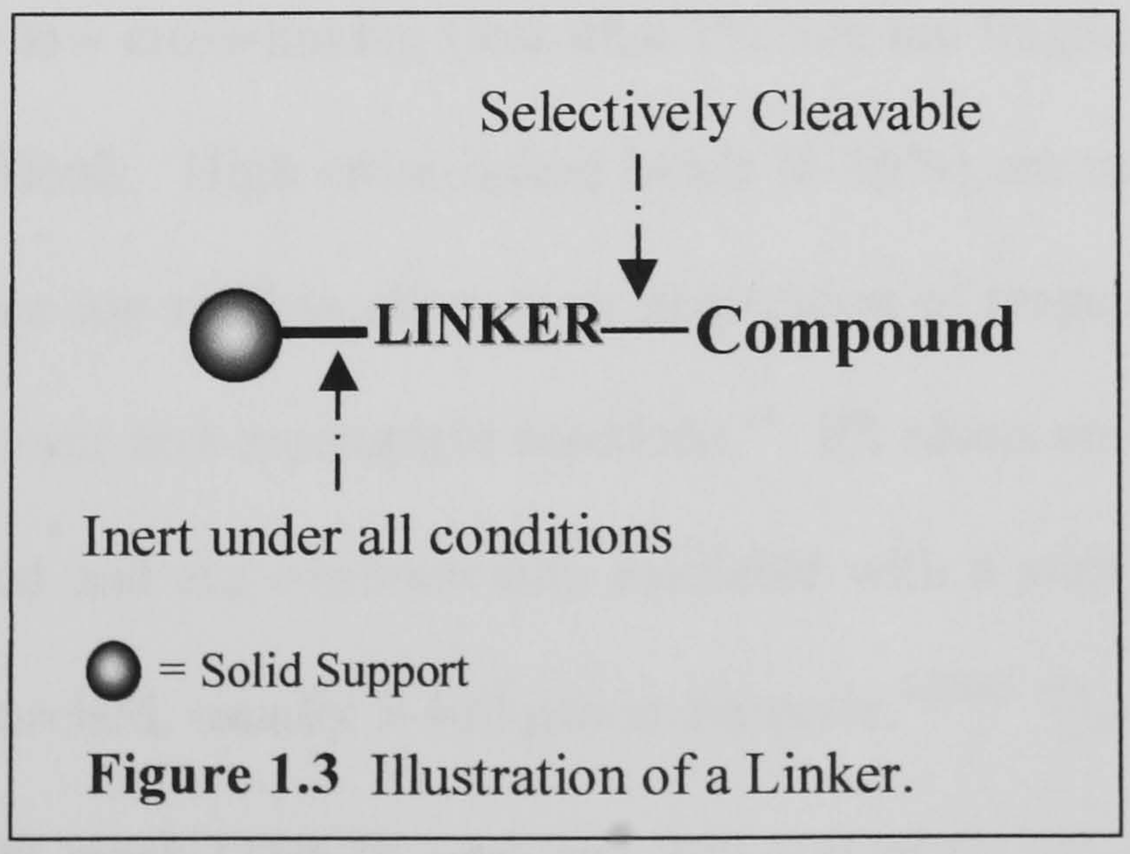


Figure 1.3 Illustration of a Linker.

1.3.2.1 Types of Solid Supports

a) Cross-linked PS Resins

Cross-linked PS resins have been used since 1963 when Merrifield first used a chloromethylated-nitrated copolymer of styrene cross-linked with 2% DVB for solid phase peptide synthesis.[14] PS resins with varying amounts of cross-linking are available for solid phase synthesis. The most frequently encountered PS resins are microporous gel-type resins and macroporous type resins.

Microporous PS-DVB resins possess a porous gel-type structure which swell up in apolar organic solvents, such as toluene, DCM, DMF, allowing easy penetration of reagents and solvents.[14] An example is PS resin with 1% DVB swells 4-6 times its original volume in DCM.[1] These resins are usually prepared by radical polymerisation of styrene and the cross-linking agent, DVB, suspended in water.[2,6,12,26] The beads produced can then be functionalised by chemical modification. Resin beads produced in this way usually have loadings which vary from batch to batch.[2] The degree of cross-linking is important as it affects the swelling, pore size and mechanical stability of the beads.[14] Beads with low cross-linking (less than 1%) are too fragile and can breakdown making filtration difficult. High cross-linked beads (8-16%) are mechanically stronger but swell little and are too rigid to allow easy penetration of reagents and solvents into the beads causing slower and incomplete reactions.[14] PS resins cross-linked with 1-2% DVB are usually used and are commercially available with a wide range of functional groups and linkers attached, usually 5-400 μm in diameter.[1,2,45c] The most common sizes of beads are 100-200 mesh (150-75 μm) and 200-400 mesh (75-37 μm).[1,2] Another method of preparing the functionalised PS-DVB resin is by adding a monomer

containing the desired functionality to the styrene/DVB mixture during polymerisation.[2,12,26] This method gives more control over loadings, particle size and swelling properties of the beads.[2,53] The loadings of linkers and functional groups attached to these resins are usually around 1 mmol/g and can reach up to 5 mmol/g.[1,2] The structure of the copolymer of styrene and 1% DVB can be seen in Figure 1.4.[54]

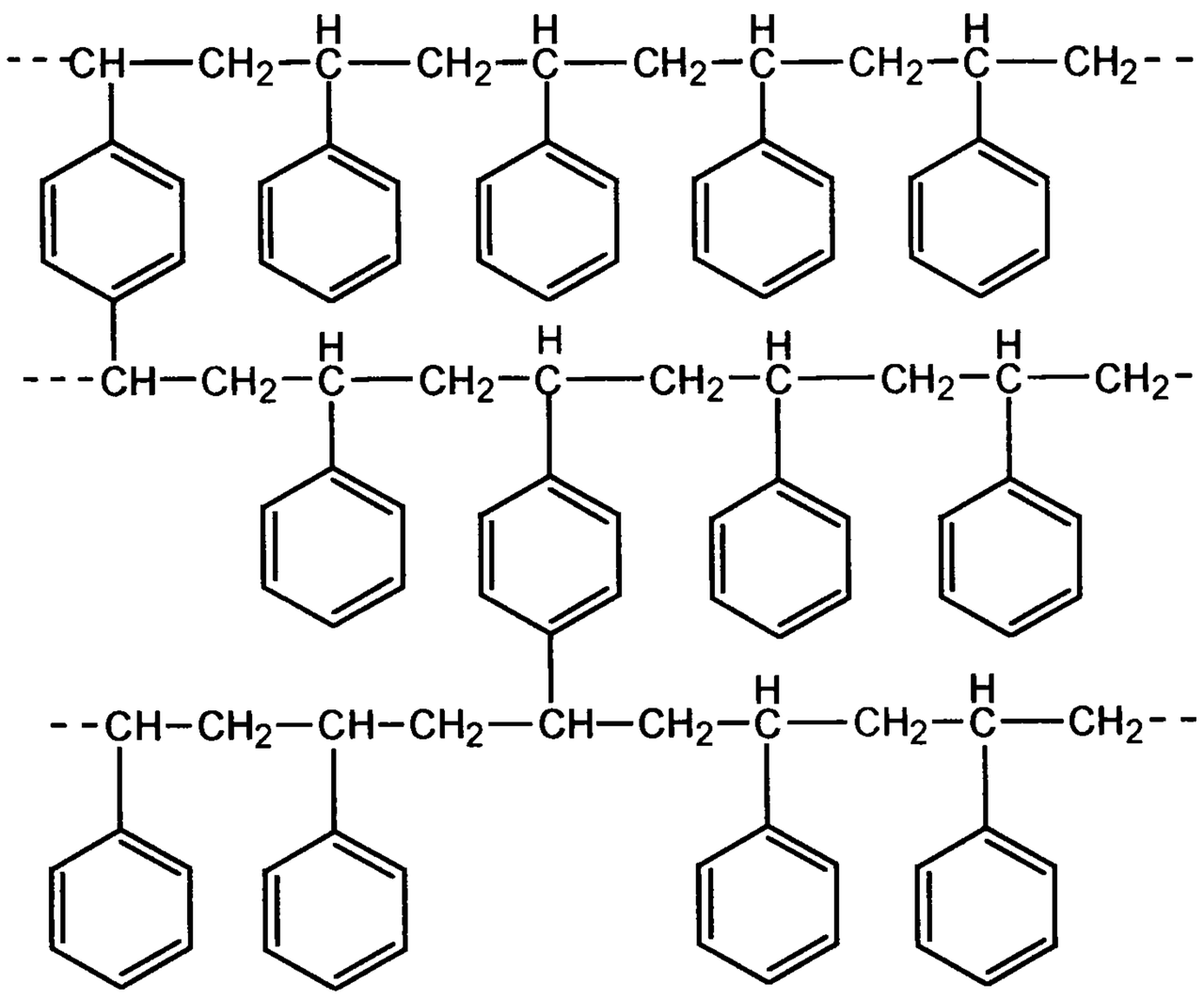


Figure 1.4 Structure of PS-DVB (1%).

Macroporous PS-DVB resins have permanent porosity, even in the dry state. They are prepared when a suspension of the dissolved monomers are polymerised in the presence of an organic solvent.[6,26,55] During the polymerisation process solvent droplets called 'porogens' are trapped within the cross-linked polymer generating large pores. The porogens are later removed by washing or evaporation. Higher amounts of cross-linking agents are added (> 10% DVB) so that the porous structure of the resulting polymer remains stable and no swelling of the resin is necessary before use.[6] Argonaut

Technologies supply Argopore® macroporous resins with a range of functionalities and linkers attached. The loading of these resins are between 0.6-1.1 mmol/g.[47b]

b) PS-Poly(ethyleneglycol) (PEG) Resins

These PEG grafted PS resins were first developed by Bayer and Rapp for solid phase peptide synthesis.[56,57] These resins under the name of TentaGel® are available commercially.[58] TentaGel supports contain around 30% of a 1-2% cross-linked PS base with long PEG side chains (around 70%) grafted onto the PS.[56] The PEG chains are immobilised by anionic graft copolymerisation of ethylene oxide onto an immobilised alcohol on the support. Figure 1.5 shows the preparation and structure of TentaGel resin.

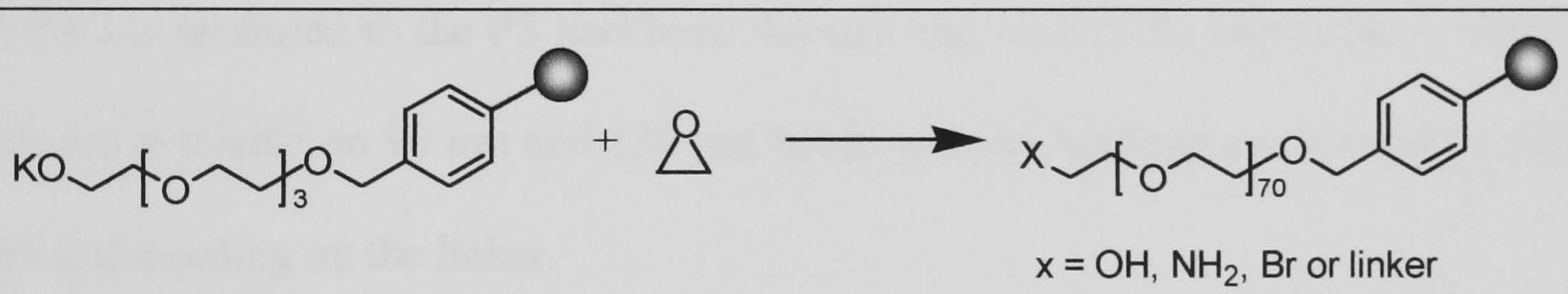


Figure 1.5 Preparation of TentaGel resin.[56]

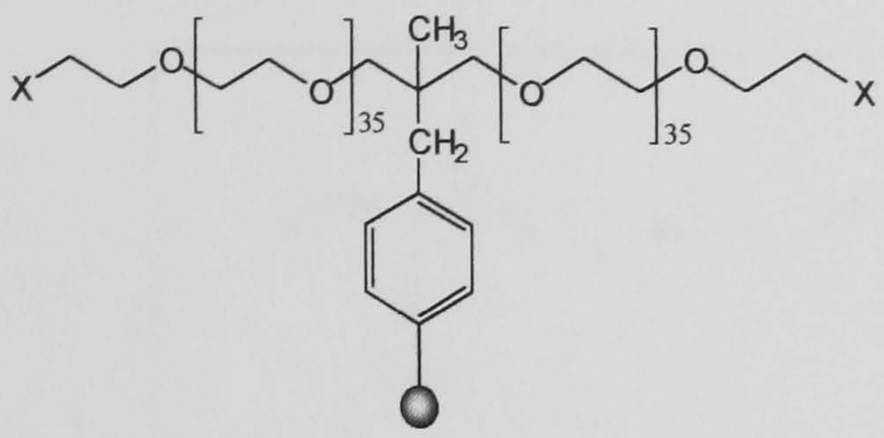
PS-PEG composite supports swell in a broader range of solvents than PS resins.[6,56,57] The PEG chains and functional groups located at the end of the chains are flexible and have mobility. They are completely solvated and behave as though they are in solution.[57] The loading of the TentaGel resins are between 0.15-0.30 mmol/g. The main problem with the TentaGel resins are that PEG is grafted to PS via a benzylic ether which can be cleaved under acidic conditions releasing PEG which contaminate products.[6,45c,47b,48] Second generation PS-PEG composite supports have been developed which are more acid stable and are available from a number of commercial sources

derivatised with a variety of linkers.[1,2,47b] These include the following and are illustrated in Figure 1.6.

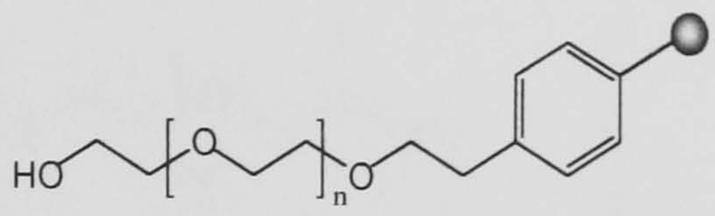
Argogel® resins (Argonaut Technologies)[47b,59] have replaced the benzylic ether linkage of PEG with an aliphatic linkage. These resins have improved acid stability and are available with a choice of functional groups and linkers. The traditional PS-PEG resins (TentaGel) are prepared by grafting ethylene oxide onto a mono-alcohol immobilised on the support. The Argonaut approach uses a diol functionalised PS intermediate which results in twice the loading capacity (0.4-0.5 mmol/g). The average diameter of these resin beads are 170 μm and the size distribution is 120-230 μm.

NovaSyn® TG Resins (Novabiochem)[2] are prepared from low cross-linked hydroxyethyl PS and 3000 to 4000 MW PEG which has been terminally functionalised. The PEG is anchored to the PS backbone through and acid stable ethyl ether. NovaSyn resins are available on 90 μm and 130 μm beads and the loadings are between 0.1-0.40 mmol/g depending on the linker.

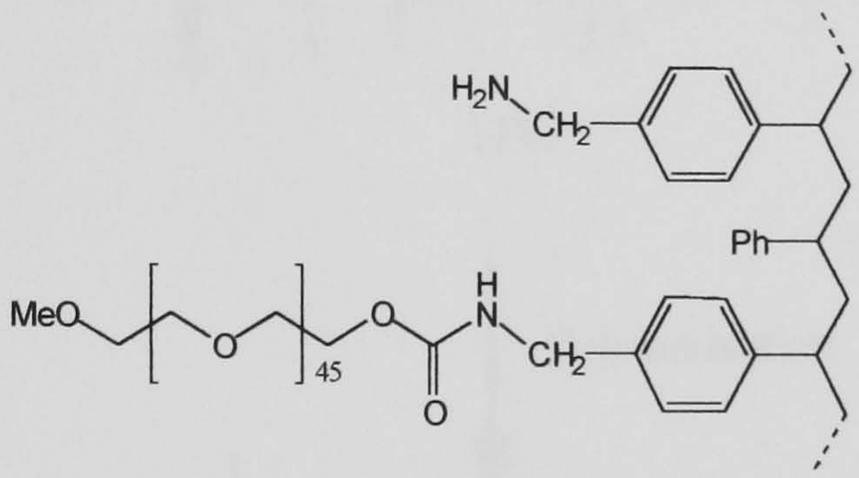
NovaGel™ Resins (Novabiochem)[2] are prepared from special high swelling aminomethyl resin which has been partially derivatised with methyl-PEG-p-nitro-phenylcarbonate. The urethane linkage between the PEG and the base PS resin is also acid stable which minimises the loss of PEG during a synthesis. If leaching of the PEG does occur it does not result in loss of substitution because the linkers are not attached to the end of the PEG chains.



(a) ArgoGel®



(b) NovaSyn®



(c) NovaGel™

Figure 1.6 Structures of the second generation of PS-PEG resins.

c) PEGA resins

PEGA (acrylamidopropyl-PEG-*N,N*-dimethylacrylamide) resins were originally developed for solid phase peptide synthesis but are now becoming used more frequently in solid phase organic synthesis.[2,60] These resins are prepared from 2-acrylamidopropyl-PEG_{800} and dimethylacrylamide cross-linked with bis-2-acrylamidopropyl-PEG_{800}. Figure 1.7 shows the preparation of PEGA resin using the three monomers.[2,6]

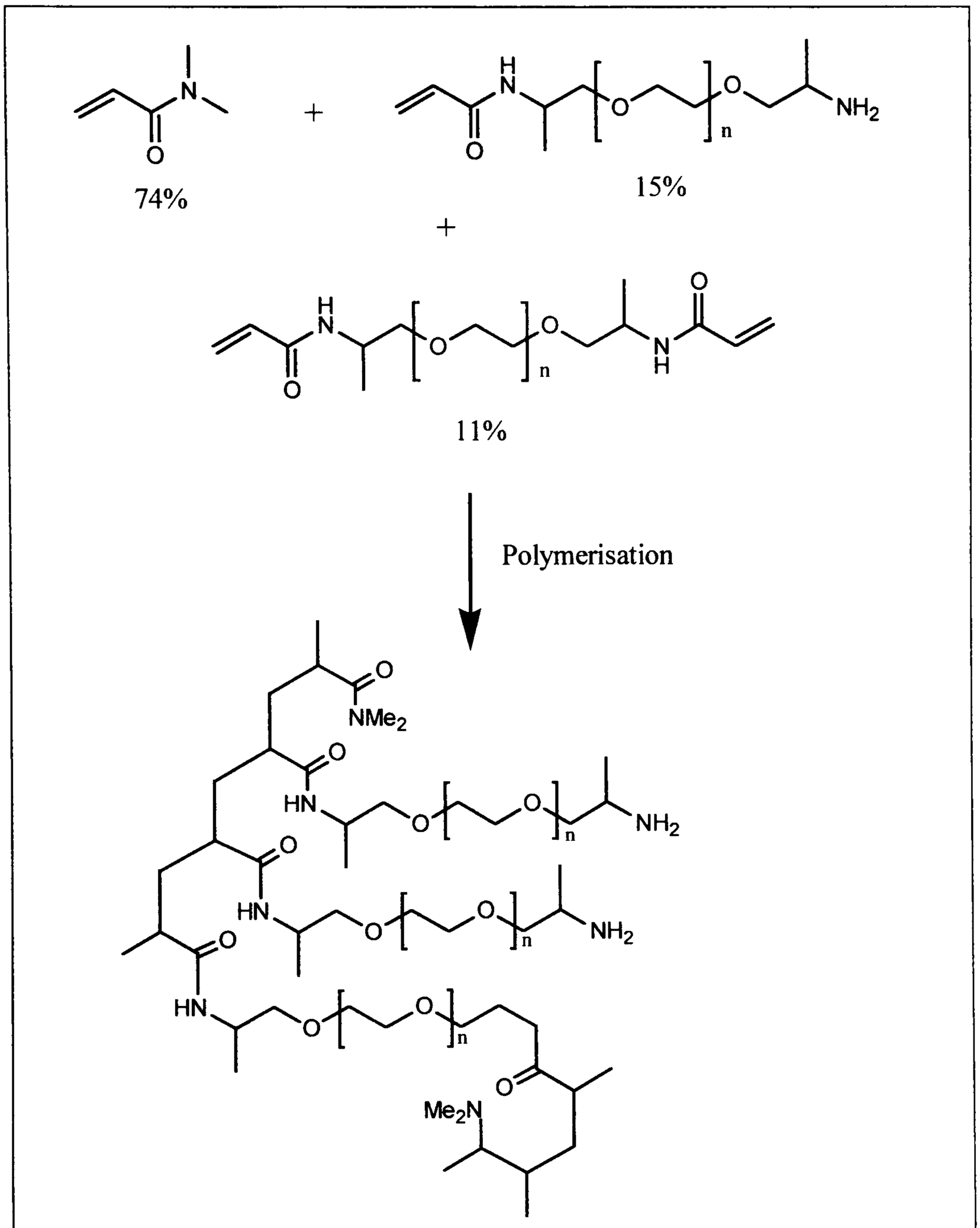


Figure 1.7 Preparation of PEGA resin.[2,6]

The advantages and disadvantages of the different solid supports used in solid phase synthesis are summarised in the following tables.

RESIN TYPE	ADVANTAGES	DISADVANTAGES
Microporous PS-DVB	Easy to handle	Not compatible with highly polar protic solvents such as water and MeOH.[2,6,45c]
	Commercially available with a broad range of functionalities and linkers.[1,2,61]	Thermal instability over 130°C.[2,25]
	Chemical stability to a wide range of reaction conditions, for example, acids, bases, reducing agents, organometallics.[2,25]	Prolonged use and stirring can cause mechanical breakdown of the resin making filtration difficult.[25]
	Can withstand temperatures of -78°C to 110°C.[2]	It has been found that during synthesis on PS-DVB resin, impurities were detected arising from the resin itself. Impurities resulted (up to 35% by weight) from trapped solvents or by-products from co-polymerisation. Can contaminate products or reduce loadings.[62]
	Swells well in DCM, THF and DMF.[2,47b]	Not suitable for use in packed columns for continuous flow processes as the resin is soft and compressable.[58]
		This resin type can suffer from osmotic shock. This can occur when the resin is placed in a solvent which makes it swell and then introduced into a solvent which makes it shrink causing high levels of stress. This can cause the resin particles to fracture or burst.[58]
		Sizes of beads vary within the same batch.[1,2,47b]

Table 1.2 Advantages and disadvantages of using microporous PS-DVB resin.

RESIN TYPE	ADVANTAGES	DISADVANTAGES
Macroporous PS-DVB	Large interior surface area which allows easy and rapid access of reagents.[26]	Lower reactivity than microporous resins.[26]
	Do not need to swell the resin prior to use.[26]	Lower loading capacity than microporous resins (0.6-1.1 mmol/g).[6,26,47b]
	Compatible with a variety of solvents including alcohols and water.[6,26]	Poor mechanical stability-breaks up easily with the formation of fines.[26] Magnetic stirring is not recommended.[47b]
	Can be used for continuous flow reactions in columns as no volume change and little swelling of the resin occurs.[6,26,58]	
	Rapid removal of solvents and reagents and ease of drying.[47b]	
	Resistance to bead cracking (osmotic shock)[47b]	
	Commercially available from Argonaut Technologies under the name of ArgoPore® with a range of attached linkers.[47b]	

Table 1.3 Advantages and disadvantages of using macroporous PS-DVB resin.

RESIN TYPE	ADVANTAGES	DISADVANTAGES
PS-PEG	The swollen PS-PEG chains are more mobile than cross-linked PS and provide a more 'solution-like environment resulting higher reaction rates.[6]	Lower loadings are observed than PS resins: (0.2-0.3 mmol/g).[57] 2nd generation resins have slightly higher loadings: (0.4-0.6 mmol/g).[2,47b]
	Compatible and swell in a wide range solvents including water, MeOH, EtOH, DMF, THF.[1,2,47b]	Because of high PEG content, the beads are sticky, adherent and difficult to dry.[6,58]
	Have a narrower size distribution than PS resins: (ArgoGel = 120-250 μm beads) (NovaSyn = available as 90 μm or 130 μm beads.)	Problems of PEG leakage due to: → Acid instability of PS-PEG linkage. Although the 2nd generation resins are much more stable.[2,6,47b] → All types of PS-PEG resins have problems with PEG leakage due to the formation of PEG peroxides by the action of light and oxygen during long term storage.[2]
	Pressure stable so suitable for both continuous flow as well as batch synthesis.[57]	Are more expensive than other commercially available resins.[1,2]
		Suffer from mechanical instability. Stirring or vigorous shaking results in significant loss of material from the resin.[27]

Table 1.4 Advantages and disadvantages of using PS-PEG resin.

RESIN TYPE	ADVANTAGES	DISADVANTAGES
PEGA	Swells in a wide range of solvents including water, DMF, DCM, THF, and methanol.[2]	Limited mechanical stability. Can cause problems with the generation of fines which can cause problems during filtration.[6]
	Has sufficient pressure stability for use in continuous flow synthesis.[57b]	PEGA resins have a low loading capacity of (0.2-0.4 mmol/g)
	Have a narrow size distribution, 50-100 mesh (300-150 μm).[2]	
	Commercially available from Novabiochem derivatised with the TFA labile 4-hydroxymethyl phenoxyacetic acid linker and the base labile 4-hydroxymethylbenzoic acid linker.[2]	

Table 1.5 Advantages and disadvantages of the use of PEGA resins.

d) Other Solid Supports

Other supports which have mainly been used for solid phase peptide or oligonucleotide synthesis include polyacrylamide based resins, silica and cellulose. The properties and limitations of these supports are shown in Table 1.6.

Although these solid supports mentioned in this thesis have been used successfully in solid phase synthesis, they do have some common disadvantages for example, most are mechanically unstable, some cannot withstand some of the harsh reagents used in organic synthesis and some are expensive. There is a need for new types of solid supports which will address the limitations of the existing support materials. The ideal solid support for solid phase synthesis would have the following properties:

1 Good Mechanical Stability

A support which is physically stable will ensure there is no breakdown of the support during synthesis. This will aid filtration and washing of the support which will improve efficiency and aid synthesis.

2 Chemical Stability

The solid support should be able to withstand a wide range of reagents and reaction conditions which will offer greater synthetic flexibility.

3 Economical to use

Solid supports which are relatively inexpensive to prepare and use would be advantageous especially when carrying out large scale reactions.

4 Easily Functionalised

The solid support should be easily derivatised with a wide range of functionalities and linkers. The loading of these groups should be sufficiently high for solid phase synthesis to be performed. A good working loading is around 1 mmol/g.[45c]

5 Easy to Handle

RESIN TYPE	ADVANTAGES	DISADVANTAGES
Pepsyn[6] A poly(dimethylacrylamide resin) prepared from N,N-dimethylacrylamide, N-acrylsarcosine methyl ester and bisacryloylethylenediamine.	More hydrophilic than PS resins and are compatible with a wide range of solvents.	Mechanically unstable. Cannot be used for continuous flow synthesis.[63]
		Low loading (0.1-0.2 mmol/g).
Pepsyn K[63-65] Pepsyn polymerised within the pores of rigid, inorganic kieselghur particles	The rigid, non-compressible framework makes it suitable for continuous flow synthesis.[63,64]	Low levels of substitution (0.1-0.2 mmol/g).
	Porous, framework of kieselghur allows rapid diffusion of reactants in and out of the gel matrix.[64]	Physical stability is poor leading to the generation of fines.[65]
		Escape of the gel during solvation can occur.[65]
PolyHipe[65] Consists of macroporous PS-DVB resin in which a poly(dimethylacrylamide) gel (Pepsyn) has been chemically bound.	PS matrix is highly branched with 90% porosity. Gives good solvent flow properties and accessibility under low pressure.[65]	Has more physical stability than Pepsyn K but still suffers from mechanical instability and breakdown from continuous use.
	Compatible with a wide range of solvents.[65]	
CPG[6] (Controlled pore glass) Composed of silica with large pore sizes (25-300 nm).	Has been used successfully in the synthesis of oligonucleotides.	Low loadings (~0.2 mmol.g).
	Has a more regular particle size and greater stability than Pepsyn K.	High cost.
		Hydrophilic nature of CPG makes water difficult to remove from the support.
Cellulose Used in the form of paper or cotton.[6,66]	Hydroxyl groups can be modified for peptide synthesis.[6]	Chemically less stable than the other supports mentioned.[6]
		Chemical modification of cellulose can increase the solubility or change the mechanical properties. This can lead to deterioration and partial loss of the support.[6]

Table 1.6 A selection of some less common supports used in solid phase synthesis.

1.3.2.2 Properties of a Linker

a) The attachment point of the linker to the solid support should be chemically stable to all conditions during synthesis and cleavage.[3,6,45c,67,]

b) The attachment point of the linker to the compound should allow reversible release of the compound under carefully controlled and selected conditions without damage to the final product.[1,45c,68]

c) The linker should allow easy and quantitative attachment of the starting materal.[3,6,68,69]

d) The linker should allow quantitative cleavage of the product.[67]

1.3.2.3 Linker Types

The choice of linker is important when carrying out solid phase organic reactions. The selection of a linker needs special consideration as the linker stability to conditions during synthesis and cleavage dictates the range of chemistries that can be used. Most of the linkers in use were originally designed for the synthesis of peptides and were bound to the support by an amide or ester bond. Cleavage of these molecules usually gave products which terminated with either carboxylic acid or amide functionalities.[5,23]

To prepare a wider range of organic molecules, alternative linkers and cleavage methods have been introduced which allow release of a broad range of other functionalities, for example, hydroxyls, phenols, amines, aldehydes and alkenes.[3,68,70] The range of linkers developed for solid phase synthesis is extensive and it has been reported that more than 200 linkers have evolved over the past 15 years.[3] Some of the most popular types of cleavage strategies involve the use of acidic or basic conditions.[1,2,3,68,71] Photocleavable and traceless linkers and linkers which induce cyclative cleavage are also available.[3,68,71]

Linkers capable of terminal variation are popular as a whole range of functional groups can be liberated from the same linker using different reagents.[2,3,68]

Examples of some common linkers and functionalised resins follow:

a) Chloromethyl linker 1

This Merrifield Resin **1** was originally developed for the solid phase synthesis of peptides.[14] Carboxylic acids and phenols can be immobilsed by nucleophilic displacement of chloride with cesium salts of the acid in DMF.[1,2,72] Alcohols and phenols can be attached using the corresponding potassium or sodium alkoxide in DMF,[2,73] thiols can also be attached.[2] Cleavage of these groups can be carried out using various reagents which adds diversity to the synthesis (See Figure 1.8).

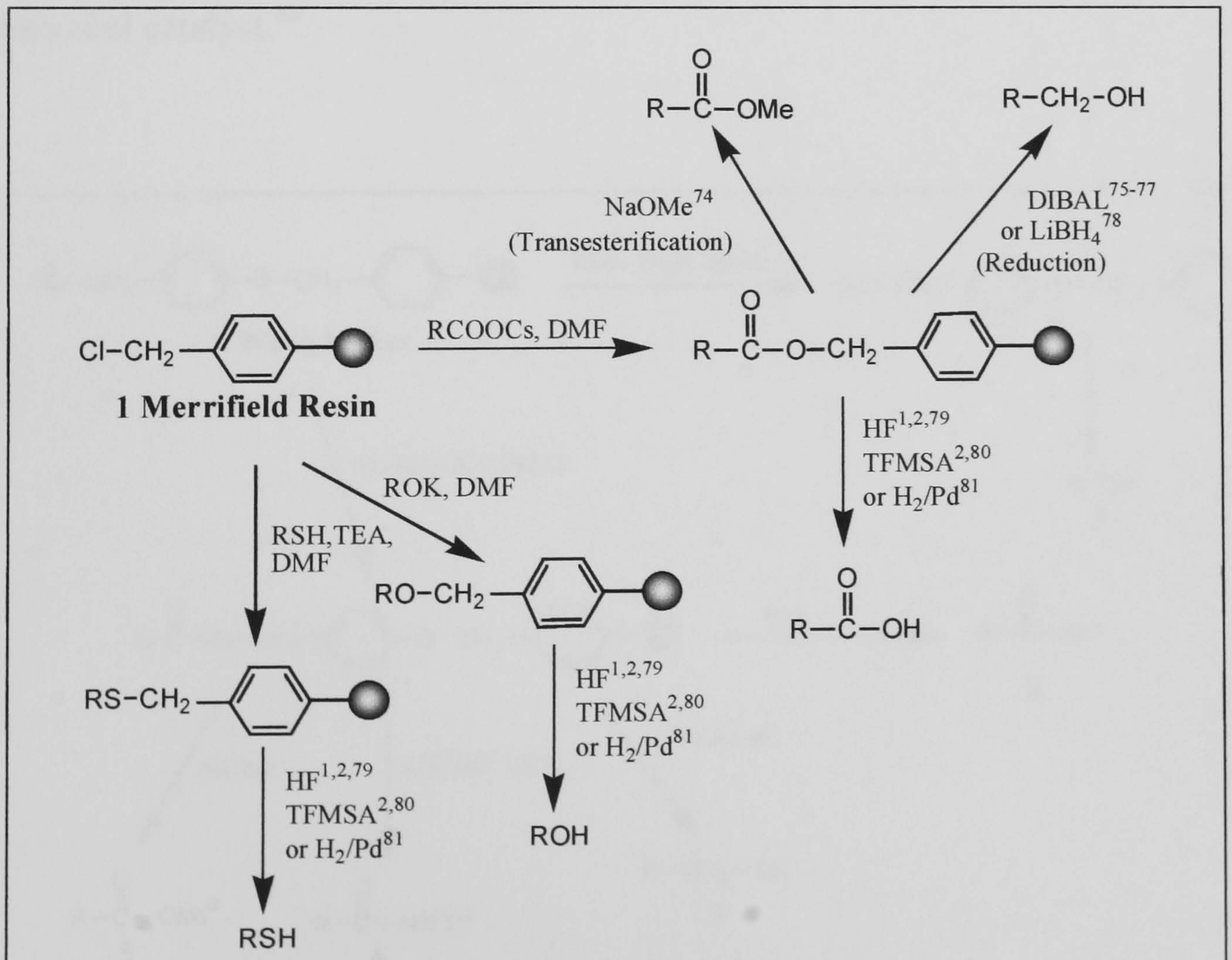


Figure 1.8 Attachment and cleavage methods using Merrifield resin.

b) Wang Linker 2

Wang (1973)[82] developed a hydroxymethylphenoxy (HMP) linker **2** for solid phase peptide synthesis. This hydroxy linker was prepared by modification of Merrifield resin[82,83] with 4-hydroxybenzyl alcohol and is a popular linker for the immobilisation of carboxylic acids and phenols[1-3,68] (See Figure 1.9).

Attachment of 4-hydroxymethylphenoxyacetic acid to aminomethyl functionalised polystyrene resin gives another form of the HMP linker.[2,84] Carboxylic acids can be coupled to the linker by the use of coupling agents such as carbodiimides.[1-3,83] Phenols are usually attached using the Mitsunobu reaction.[85,86] Cleavage of these groups to generate the carboxylic acid **4** and phenols **3** respectively is commonly carried out using 50% TFA in DCM.[1,2,45c,83] Cleavage with NaOMe produces methyl esters **5.**[87] Tertiary amides **6** can be generated upon cleavage with a secondary amine in the presence of a Lewis acid catalyst.[88]

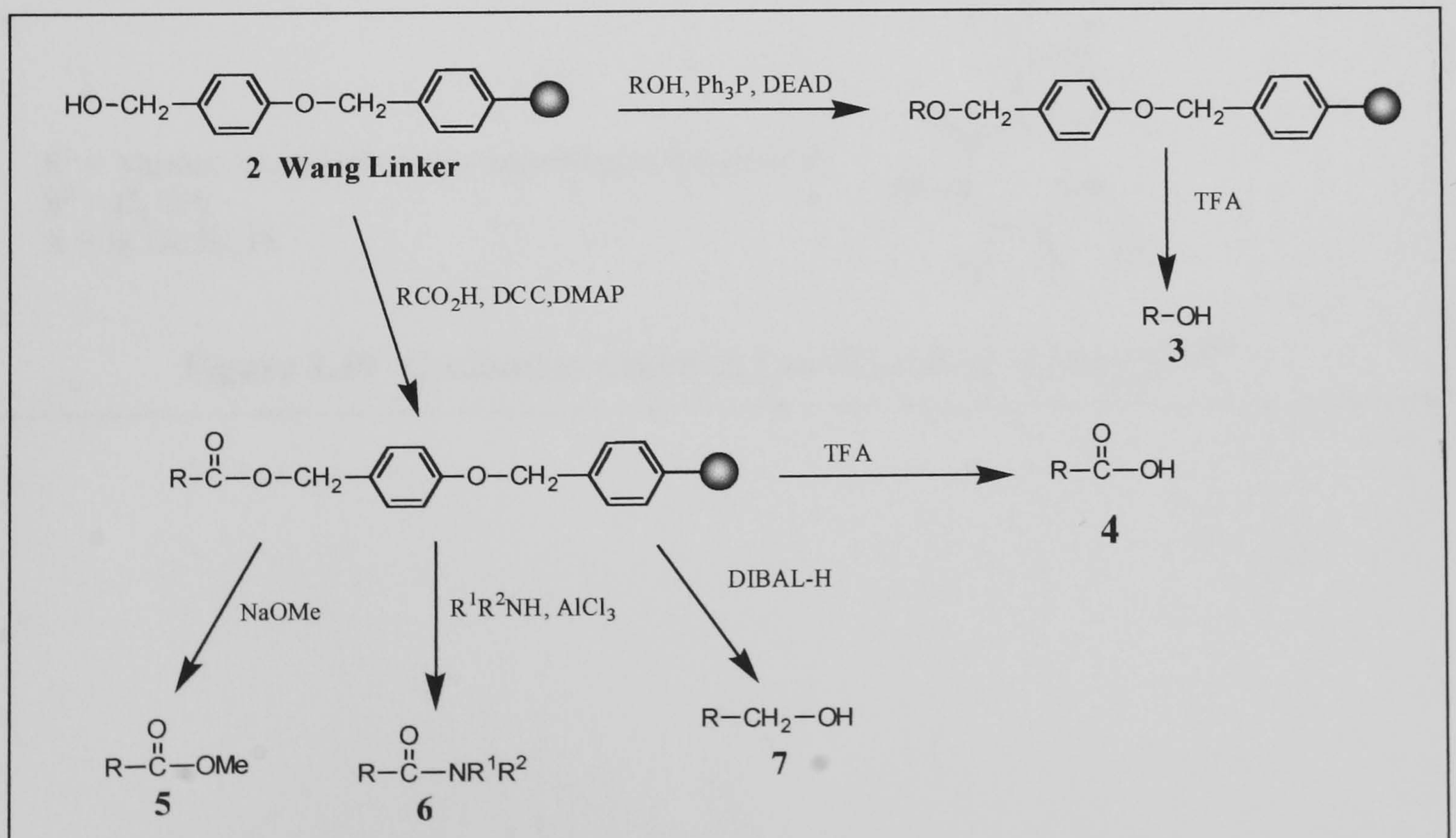


Figure 1.9 Attachment and Cleavage Methods for the Wang Linker.

Cleavage of the ester can also be carried out using DIBAL-H to generate the alcohol **7**.[89] The Wang linker has also been used for cyclative cleavage. An example of a cyclisation mediated cleavage is illustrated in the preparation of 1,4-benzodiazepine-2,5-diones **11** using Fmoc amino acid derivatised Wang resin **8** and protected anthranilic acids **9** (2-aminobenzoic acids).[90] (See Figure 1.10). Base promoted cyclisation of the amino acid anthranilate intermediate **10**, after Fmoc removal, forms the 1,4-benzodiazepine-2,5-dione **11** and releases it into solution.[90] This type of cyclative strategy results in very pure products as only the desired cyclised material is released. In the preparation of the benzodiazepin-2,5-diones yields are 46-80% with purities in the range of 81-99%.[90]

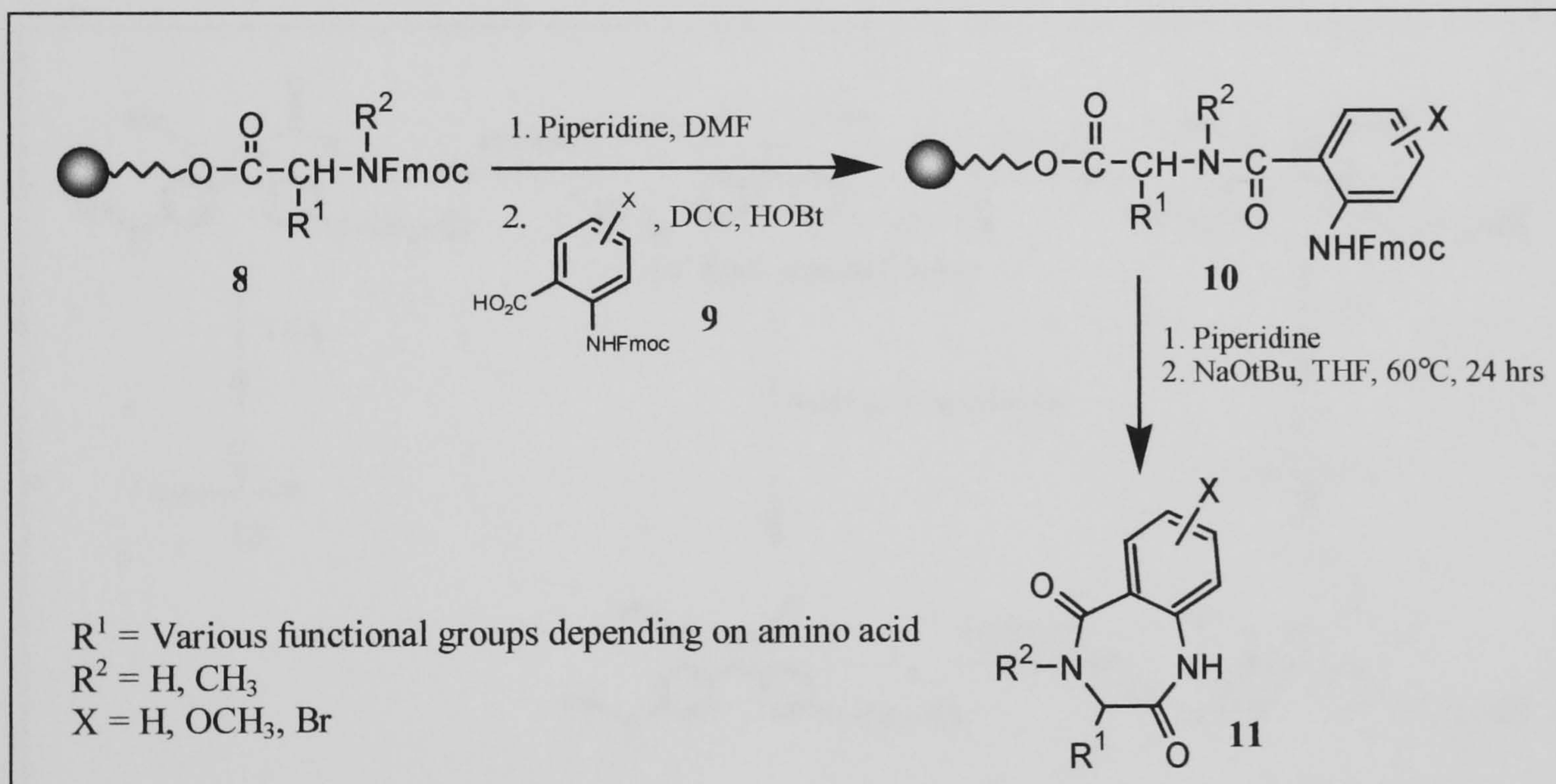


Figure 1.10 Cyclisation mediated cleavage using Wang resin.[90]

c) Rink Amide Linker 12

The rink linker **12** (Figure 1.11) is one of the most common linkers for the preparation of primary carboxamides **13** and sulfonamides **14**.[91] The Rink linker can be obtained attached directly to PS-DVB resin *via* an ether bond or it can be attached to aminomethyl functionalised resins via an amide bond.[2] The resin bound amino group, after removal of the Fmoc protecting group, can be acylated using a range of common acylating agents such as DCC/DMAP or PyBOP®.[1,2,92] Reductive alkylation of the rink amide before acylation, upon cleavage generates secondary carboxamides[93] **15**. Cleavage is carried out using TFA.[2,68,91]

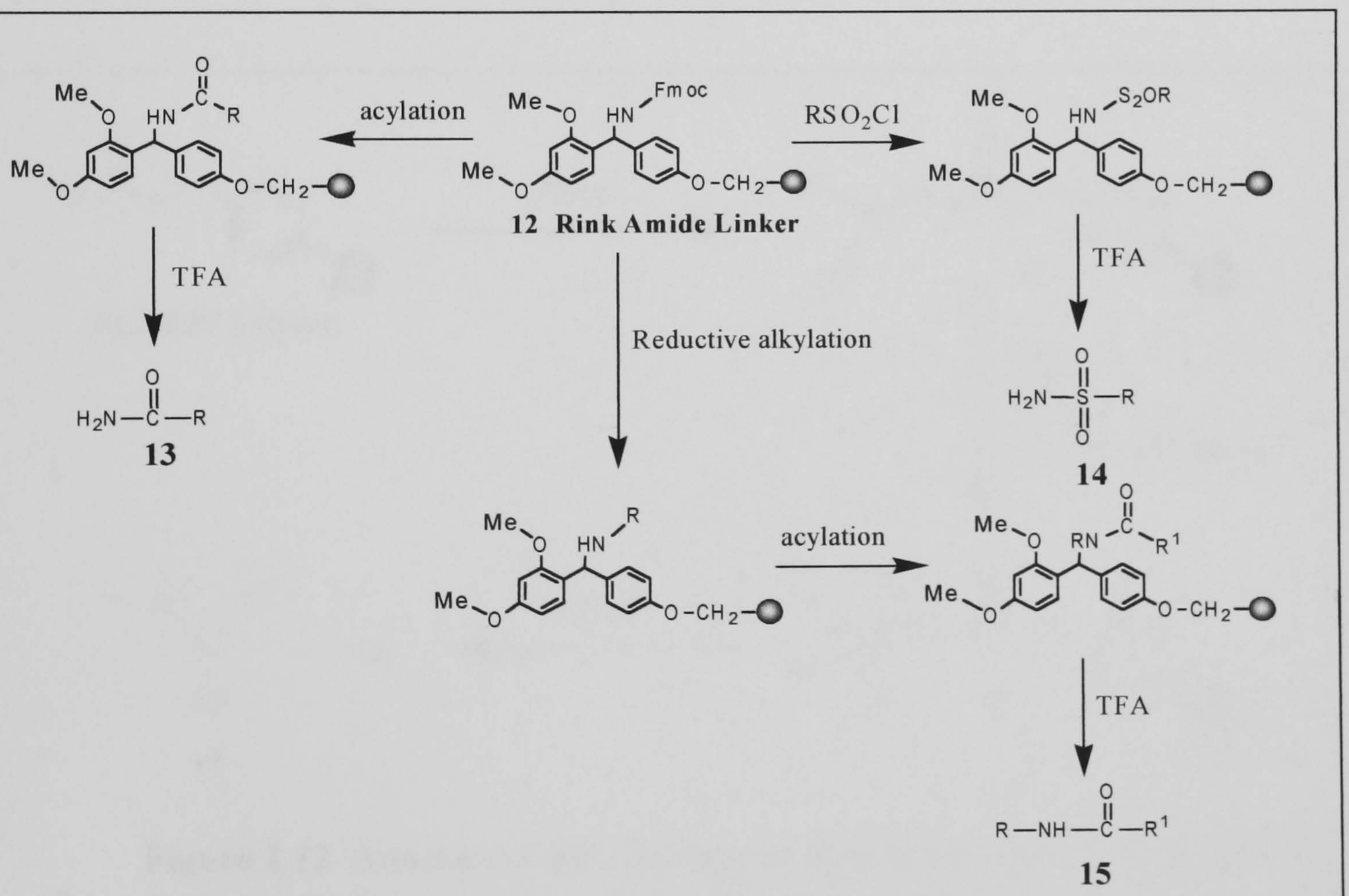


Figure 1.11 Attachment and Cleavage Methods for the Rink Amide Linker.

d) REM Linker 16

The REM linker **16** was developed in 1996 for the immobilisation of secondary amines.[94] This linker is prepared by the attachment of acryloyl chloride to hydroxymethyl PS.[94,95] Michael addition of the secondary amine onto the resin bound acrylate **16** (Figure 1.12) gives a resin bound tertiary amine **17**. Quaternisation of the tertiary amine by alkylation with a number of reactive halides (eg. methyl, allyl and benzyl bromides or iodides[3]) produces a resin bound quaternary ammonium salt **18**. Mild cleavage with DIPEA by Hofmann elimination gives the corresponding tertiary amine **19** and the regenerated linker **16**. Since the linker is REgenerated after cleavage and is functionalised by Michael addition, the resin is referred to as the REM resin.[94,95]

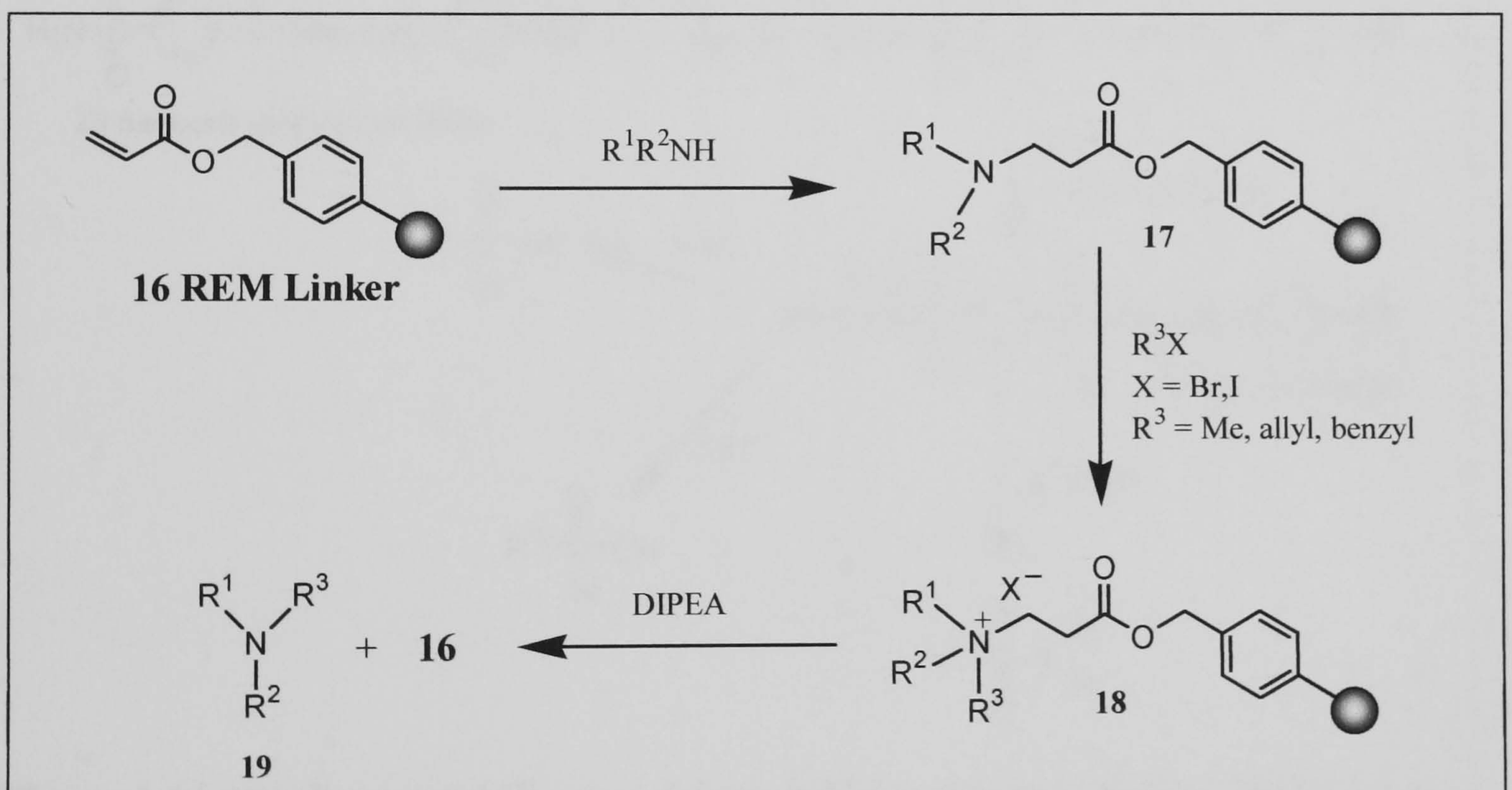


Figure 1.12 Attachment and Cleavage of Amines attached to REM Linker.

e) Kenner's Safety Catch Linker 20

This linker **20**, prepared by attaching 4-sulfamidobenzoic acid to aminomethylated polystyrene, was developed in 1971 and is useful for the immobilisation of a variety of carboxylic acid derivatives[96] (See Figure 1.13). Acylation with carboxylic acids produces a resin bound sulfonamide **21** which is completely stable to acidic and basic conditions. The linker can be activated by alkylation with either diazomethane or iodoacetonitrile.[96-98] After activation **22**, cleavage is possible with thiols,[99] hydroxide ion[98] or primary or secondary amines[97,98,100] to generate thio-esters **23**, carboxylic acids **24** and carboxamides **25** respectively.[2,97,98]

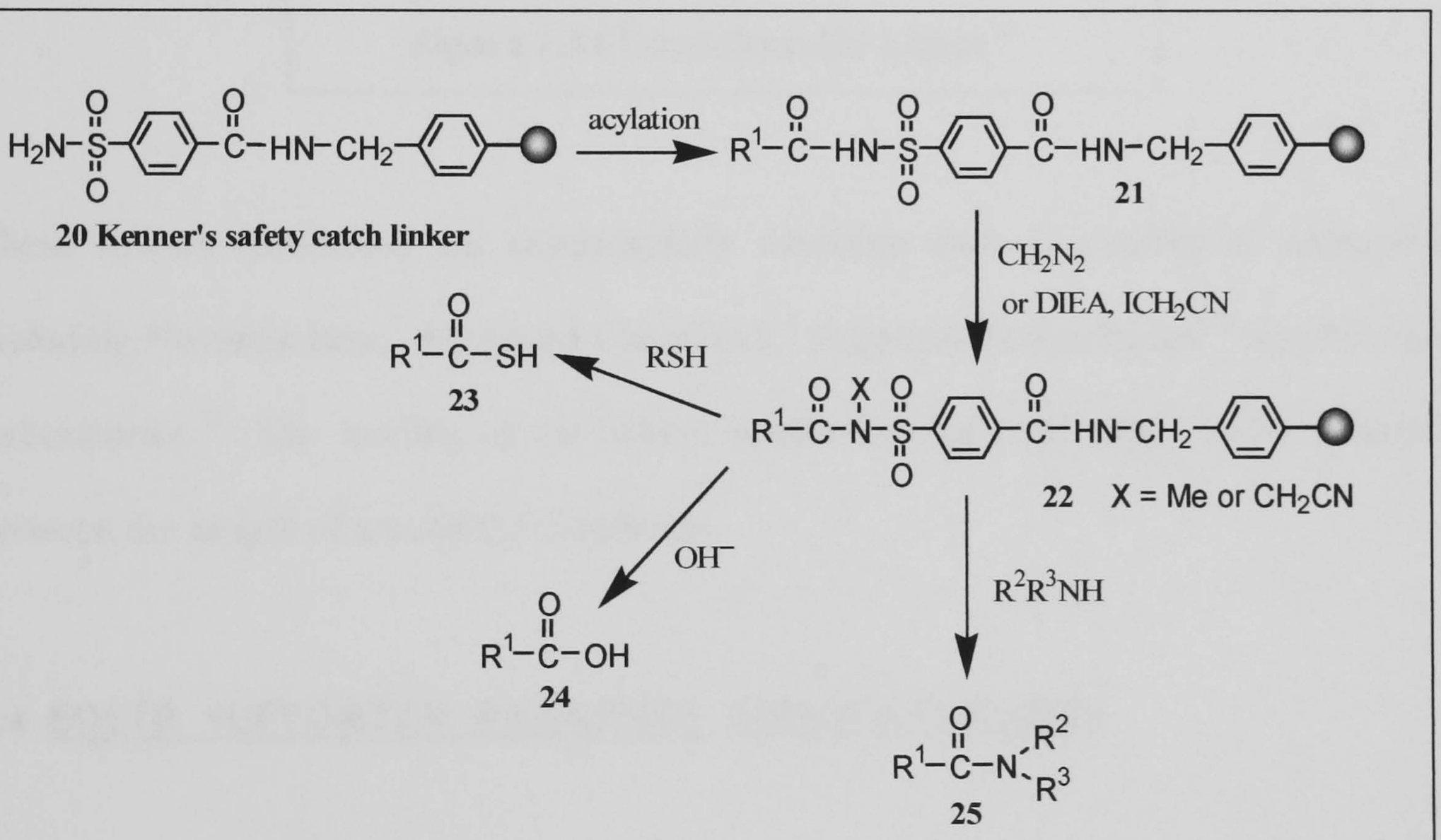


Figure 1.13 Attachment and Cleavage Methods Using Kenner's Safety Catch Linker.

f) Photocleavable Linker 26

Affymax have developed a range of photolinkers which are used for the immobilisation of carboxylic acids.[101,102] One example is the hydroxyethyl photolinker **26** (See Figure 1.14) attached to aminomethylated polystyrene resin and is used to immobilise

carboxylic acids.[2,102] Photocleavable linkers have proved valuable for the solid phase synthesis of oligomers and small molecules and are stable to a wide range of chemical conditions. Molecules are easily cleaved from the photolinker under neutral conditions using UV light at 365 nm.[101,102] They are particularly useful for the preparation of pH sensitive compounds.[1]

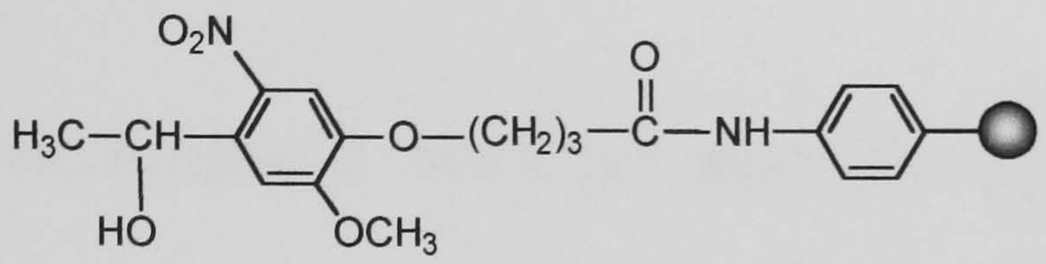


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Figure 1.14 Photocleavable Linker.[2]

These linkers mentioned are commercially available from a number of companies including Novabiochem,[2] Advanced ChemTech,[1] Argonaut Technologies[47b] and Polymer Laboratories.[53] The loading of the linkers attached to the solid supports are available between the ranges of around 0.1-5 mmol/g.

1.4 SOLID SUPPORTED REAGENTS AND SCAVENGERS

1.4.1 Limitations of Solid Phase Organic Synthesis

Despite the obvious advantages of solid supported organic synthesis, there are some limitations which come with using this method which include:

a) Reactions can be slow on the solid phase compared with the same reaction in solution.[9]

b) It is difficult to monitor reaction progress during SPOS. Conventional and reliable monitoring techniques such as TLC and NMR cannot be used.[9] There are a number of methods available for monitoring solid phase reactions, for example, colorimetric assays,[70] FT IR,[103] elemental analysis[104] and Gelphase NMR[105] but it has been reported that they do not provide the same quality of analysis as the traditional monitoring techniques.[9] Most of the methods used are also more time consuming and less sensitive.

c) Additional steps are required to attach and detach the materials from the solid support.

d) The solid support and linker type limits the possible chemistry which can be performed.[23]

e) The optimisation involved from transferring the reaction from solution to solid phase can be long and tedious.[9]

Solution phase synthesis does not have these disadvantages but a major drawback with synthesis in solution is the difficulty encountered in purifying the end products. However, the development of polymer bound reagents and scavengers (mentioned briefly in Section 1.1) overcome this problem.

1.4.2 Benefits of Using Solid Supported Reagents and Scavengers

There are important advantages of using solid supported reagents and scavengers, these are detailed below:

a) Reaction work up and product isolation after synthesis involves simple filtration of the used solid supported reagent and/or scavengers and evaporation of the solvent.[2,106]

b) Excess immobilised reagents can be used to drive reactions to completion. Any unreacted or spent reagent can then be recovered by filtration.[2]

c) Resin bound toxic or hazardous compounds can be handled safely once immobilised onto the solid support.[2]

d) Polymer bound catalytic reagents can be easily removed and re-cycled.[2]

e) Reagents which are incompatible in solution, when immobilised can be used simultaneously.[2,9]

f) Immobilised scavengers can be used in automated parallel synthesis. After each synthetic step the reaction mixture can be passed through a bed of immobilised scavenger resulting in simple and rapid purification.[107]

1.4.3 Types of Solid Supports used to Immobilise Reagents and Scavengers

Microporous PS cross-linked with 1-2% DVB, macroporous PS resin and silica have been used as base resins for the attachment of reagents and scavengers.[2,47b,107] Many of these supports have not been specifically designed for use with reagents or scavengers and have properties which can reduce their performance.[107] The advantages and disadvantages of using these supports as scavengers and immobilising reagents are outlined in Table 1.7.

RESIN TYPE	ADVANTAGES	DISADVANTAGES
Microporous gel-type PS resin (1-2% DVB)	Commercially available from a number of sources immobilised with a range of reagents, scavengers and catalysts: Novabiochem,[2] Argonaut Technologies[47b] and Advanced ChemTech.[1]	The ability of this resin to swell considerably can reduce scavenging capacity and can cause problems when using volume limited flow-through apparatus.[107]
		For other disadvantages see Table 1.2.
Porous Silica	Prepared form purified 99.995% porous silica microspheres.[107]	See Table 1.6.
	Low metal content, low acidity and a narrower size distribution (20-80 μm) than commercial grade silica.[107]	
Macroporous PS-DVB resin	Only minimal swelling of this resin observed so can be used for small scale reactions and when packing into continuos flow apparatus.[107]	See Table 1.3.
Commercial Silica[107]	Does not swell in solvents.	Broad particle size distribution can result in non-uniform flow rates.
		The generation of fines can reduce filtration and contaminate final products.
		Often contains surface metal ions which can catalyze unwanted reactions.

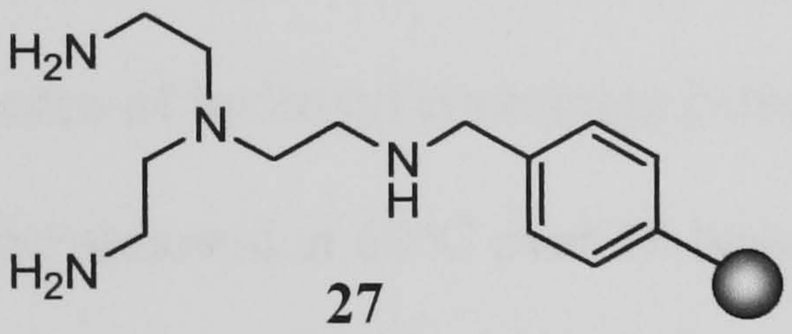
Table 1.7 The advantages and disadvantages of supports for the immobilisation of scavengers and reagents.

1.4.4 Selected Examples of Some Commercially Available Immobilised Scavengers and Reagents

1.4.4.1 Scavenger Resins

a) Tris(2-aminoethyl)amine Resin 27

This nucleophilic resin (See Figure 1.15) has been used to scavenge and remove acid chlorides, sulfonyl chlorides, isocyanates, isothiocyanates and acids.[2,13,47b] The quenching reaction is normally carried out at room temperature in polar solvents such as DCM, DMF and THF with the reaction complete between 0.5-4 hours.[2] 1.5-3 equivalents of the resin relative to the electrophile to be quenched is added. With acid and sulfonyl chlorides 3-6 equivalents are added to neutralise the HCl generated unless an additional base is used.[2,47b]



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Tris(2-aminoethyl)amine resin[13]

Commercial Source	Base Resin	Loading (mmol/g)	Mesh size
Novabiochem	PS-DVB (2%)	1.4-1.8	200-400
Argonaut	PS-DVB (1%)	1.0-1.7	100-200
Adv.ChemTech	PS-DVB (1%)	0.6-1.0	100-200

Figure 1.15 Tris(2-aminoethyl)amine scavenger resin.

An example of a reaction using the scavenger resin **27** is the reaction between an isocyanate and an amine shown in Figure 1.16.[13] Excess of the amine is reacted with the iosocyanate, the scavenger **27** is added which reacts and quenches the excess amine, filtration and evaporation of the solvent then gives the product. Both the reaction and the quenching procedure can be monitored by TLC and ^{1}H-NMR and show good purity.

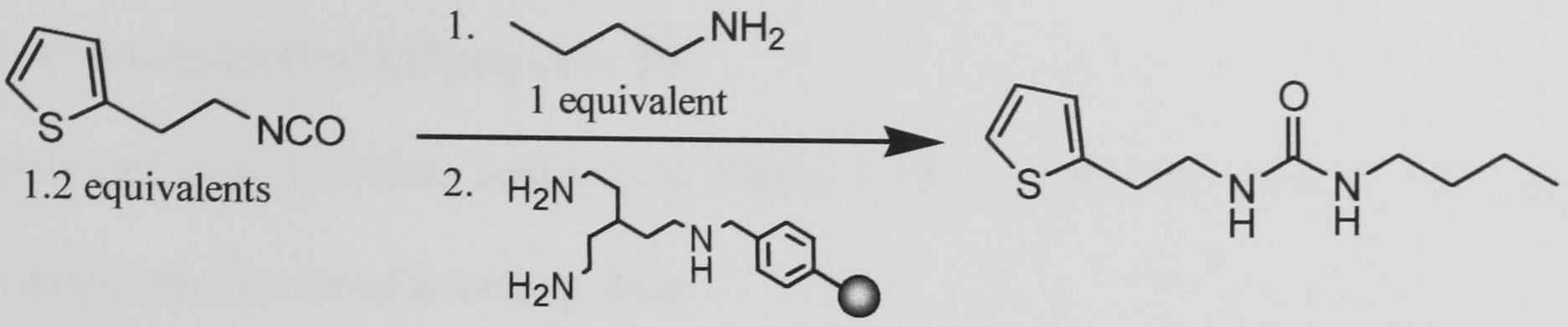
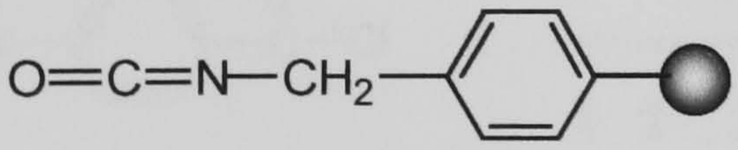


Figure 1.16 Synthesis using tris(2-aminoethyl) scavenger resin.[13]

b) Methyl isocyanate resin 28

This electrophilic scavenger (see Figure 1.17) removes nucleophiles such as amines and alkoxides without releasing any by-products.[2,13] 1.5-3 fold excess of the scavenger is added relative to the nucleophile to be removed. The reaction with primary and secondary amine occurs rapidly at room temperature over 1-3 hours. Alcohols are unreactive under these conditions therefore the amine can be selectively removed in the presence of hydroxyl containing compounds.[2] Aromatic amines are more unreactive and can be removed at 60°C over 18 hours. An example of the use of this scavenger resin is the reaction outlined in Figure 1.11 but the amine is used in excess instead of the isocyanate. The methyl isocyanate scavenger can then remove the excess amine.[13]



28

Methyl isocyanate resin[13]

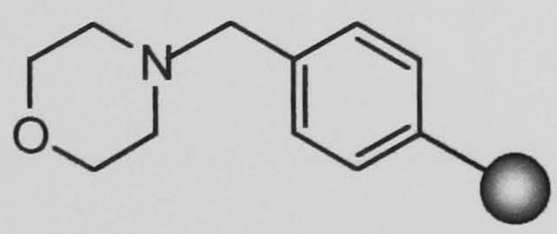
Commercial source	Base Resin	Loading (mmol/g)	Mesh size
Novabiochem	PS-DVB (2%)	1.0-1.3	200-400
Argonaut	PS-DVB (1%)	1.0-1.7	100-200

Figure 1.17 Methyl isocyanate scavenger resin.

1.4.4.2 Immobilised Reagents

a) Morpholinomethyl polystyrene 29

This polymer bound tertiary amine (see Figure 1.18) is useful for a variety of reactions which involves the use of a tertiary base.[2,13]



29

Figure 1.18 Morpholinomethyl polystyrene.

The need to separate the amine salts at the end is eliminated.[2] This reagent can be used in conjunction with the tris-amine scavenger and the isocyanate resin for the preparation of amides.[13] This reaction is exemplified in Figure 1.19 where a 81% yield was obtained[13] Novabiochem supply this resin with a loading of 3.2-3.8 mmol/g with the base resin consisting of PS with 2% DVB cross-linking with a size of 200-400 mesh.[2]

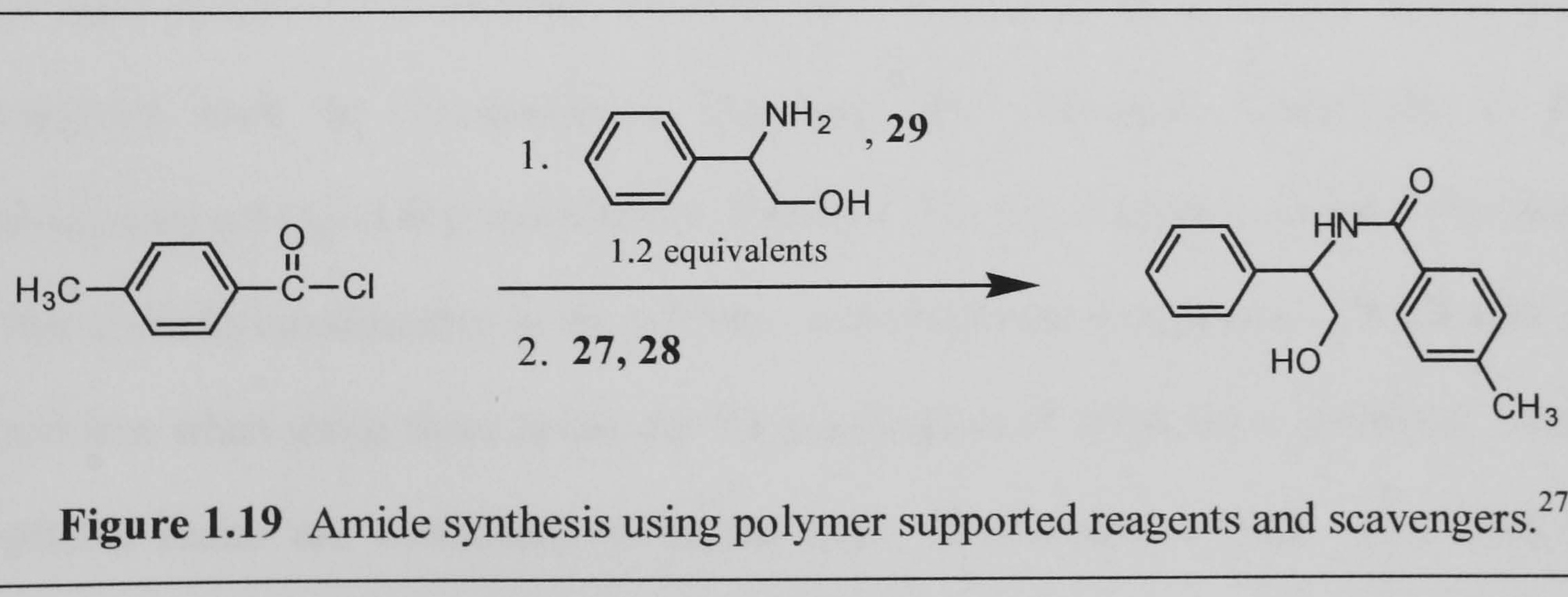
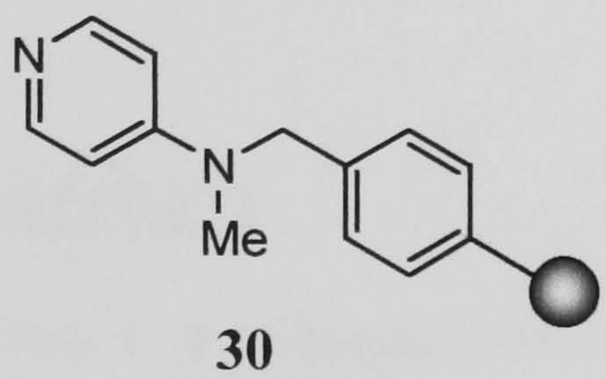


Figure 1.19 Amide synthesis using polymer supported reagents and scavengers.[272]

b) Polystyrene immobilised DMAP 30

This acylation catalyst (See Figure 1.20) has been immobilised onto 4% crosslinked (DVB) polystyrene resin and has been used to accelerate the esterification of tertiary alcohols.[47b] Usually 0.1 equivalents of the immobilised catalyst is added to the reaction mixture overnight at 110°C. The resin beads are 30-80 mesh with loadings in the range 1.1-1.8 mmol/g.[47b]



30

Figure 1.20 Polymer bound DMAP.[47b]

1.4.5 Properties of an Ideal Solid Support for the Immobilisation of Reagents and Scavengers

The solid supports used to immobilise scavengers and reagents have their limitations. The most common commercially available base resin used by a number of chemical companies such as Novabiochem, Argonaut and Advanced ChemTech is the microporous gel-type DVB cross-linked PS resin. The major disadvantages of this resin is that it swells considerably in the solvents used for chemical synthesis. This is seen as a problem when using these resins for the purification of small scale reactions. Non-swellable resins are considered to be the best. Some non-swelling resins such as macroporous PS resin and silica have been developed and used but suffer from mechanical stability. It is recommended by the manufacturers that the resins should be

agitated gently.[2,47b] This is because conventional stirring techniques such as magnetic stirring would degrade the resins.

The ideal base resin for the immobilisation of scavengers and reagents should have the qualities listed below:

1 Non-swellable in common organic solvents

2 High loading of functional groups

3 Mechanical and chemical stability

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2 THE USE OF INSOLUBLE SUPPORTS IN DRUG DELIVERY

2.1 TYPES OF DRUG DELIVERY

There have been a number of methods of drug delivery into the body. Traditional methods of drug delivery involve the use of injections or oral formulations such as tablets.

Other modes of drug delivery include:

a) Transdermal drug delivery where the drugs diffuse through the pores of the skin.[1] An example of this type of drug delivery is where the hormone estradiol is given transdermally to treat post-menopausal symptons.[2]

b) Respiratory drug delivery which involves the delivery of drugs to the lungs such as dry powder aerosols,[2] for example, the use of inhalers to treat asthma.[3]

c) Intranasal delivery which administer powders in the form of an aerosol based nasal spray and the drug is absorbed through the nasal mucosa.[2,4]

d) Needleless injections where medicines and vaccines are delivered transdermally without breaking the skin and without causing pain.[3]

2.1.1 Disadvantages of Injections and the use of Tablets

Injections using hypodermic needles and the use of tablets administered orally are still the most common modes of drug delivery but suffer from some disadvantages.

2.1.1.1 Injections

a) Injections can cause discomfort and pain to the patient.[3]

b) High doses of drugs cannot be injected into the body at one time.[1]

c) After an intravenous injection a high concentration of drug in the bloodstream results which can cause toxic side effects.[1]

d) During administration, there is a risk of introducing harmful microbes into the patient.[3]

2.1.1.2 Oral Delivery

a) Tablets can be difficult to swallow

b) Only drugs which can survive the acidic environment and the digestive enzymes present in the gastrointestinal tract can be used.[3]

Another major disadvantage of these two methods of oral delivery is that after administration there is a rise of drug levels in the blood which can create toxic side effects.[5] This level then decreases until the next administration. There is a need for the drug to remain constant between a desired maximum and minimum level in the blood for an extended period of time.[5] This can be achieved by controlled drug delivery.[5]

2.1.2 Controlled Drug Delivery

Controlled drug delivery systems involve the combination of a drug or bioactive agent with a polymer.[5,6] When this system is placed in the body the material is released from the polymer at a pre-determined rate.

The advantages of controlled drug delivery are:[5]

a) The maintenance of drug levels within the desired range.

b) Fewer administrations are necessary.

c) Increased patient compliance.

d) The potential of under or overdosing can be reduced.

2.1.2.1 Types of Controlled Drug Delivery

Some of the areas where polymers have been used include the following:

1 Oral controlled release.[5]

2 Sustained release injections.

3 Polymeric implants.[1]

4 Transdermal drug delivery.[5]

2.1.3 Controlled Release Mechanisms

There are a number of mechanisms by which drugs are released from the drug delivery system. These include:

2.1.3.1 Diffusion Controlled Systems

Diffusion occurs when the drug passes through the polymer and comprises of two types.

a) Reservoir Type

The reservoir delivery system consists of a non-degradable polymer with a spherical, cylindrical or slab like form. The core consists of a drug in powdered or liquid form.[5] The drug then slowly diffuses through the polymer.[2,5,7] Some of the problems encountered with this system is that the implant must be removed after use as the polymer does not degrade and if polymer membrane accidentally ruptures, the drug will all be released suddenly which could prove toxic to the patient.[7]

b) Matrix Type

This type of diffusion controlled system comprises of a polymer and drug mixed together to from a uniformly distributed homogeneous system.[5] The drug is then released by diffusion from the polymer matrix into the body. The advantages of this system is that there is no risk of accidental release of a high concentration of drug. The only drawback

is that the release rate usually decreases over time as the drug in the middle of the polymer has a longer distance to travel before release.[5]

2.1.3.2 Solvent Activated Systems

Two types of solvent activated systems have been developed:

a) Osmotically Controlled release[2,7]

This drug delivery system possesses a polymer with a semi-permeable membrane. When placed in the body the external fluid moves across the membrane and into the system where the drug is in high concentration. The inward movement of the fluid forces the dissolved drug out of the device.[7]

b) Swelling Controlled release[2,5,7]

Swelling controlled release systems start dry and when placed in the body absorb fluids and swell without dissolving.[5] This polymer type consists of hydrophilic macromolecules cross-linked to form a three dimensional network.[7] Swelling increases the size enabling the drug to diffuse through the swollen network into the body.

These are examples of environmentally responsive systems which only release the drugs when placed in an appropriate biological environment.[5]

2.1.3.3 Biodegradable Systems

These involve the use of polymers which degrade within the body naturally.[5] There are three approaches to the biodegradable systems.[5]

1 Erosion of the polymer surface which releases the physically entrapped drug.

2 Cleavage of covalent bonds between the polymer and drug when placed in the body. Chemical hydrolysis or enzymatic cleavage usually releases the drug. The drug can be linked directly to the polymer or *via* a spacer group.

3 Diffusion controlled release of the physically entrapped drug out of the polymer. The polymer then degrades after the drug has been depleted.

2.1.4 Types of Polymers used in Drug Delivery

A range of synthetic and natural polymers have been investigated for use in drug delivery.[1,2,5] Biodegradable and non-biodegradable polymers have been examined. The major disadvantages of using non-biodegradable polymers in drug delivery is that they have to be removed from the body after use.[7] Polymeric implants prepared using non-degradable polymers need to be surgically removed. This introduces the possibility that some non-degradable material may be left behind which could cause problems for the patient.[7]

Biodegradable polymers do not have this problem as they degrade naturally within the body. It is essential that all biodegradable products as well as their metabolites are non-toxic, non-carcinogenic and non-teratogenic.[8]

Examples of some polymers investigated are:

2.1.4.1 Natural Polymers

The natural polymers which have been used for drug delivery include collagen, cellulose and chitosan and these are capable of undergoing degradation in the body.[1]

2.1.4.2 Non-biodegradable Synthetic Polymers[5]

Polyvinyl alcohol

Polyethylene glycol

Polyacrylic acid

2.1.4.3 Biodegradable Synthetic Polymers

Polylactides[5]

Polyglycolides[5]

Polyanhydrides[5]

Poly (lactide-co-glycolides)[9]

Polyphosphoesters[10]

The most common form of these biodegradable materials are microparticles which have been used in oral delivery and injected drug delivery systems.[5]

Alkermes of Massachusetts have developed a number of sustained release microsphere formulations designed to reduce the need for frequent dosing.[9] ProLease® is one of their patented injectable systems. This system is designed for use with complex and fragile bioactive molecules. The polymer consists of Poly(lactide-co-glycolide) which is used for its biocompatability and biodegradability. Polymer microspheres containing the drug are prepared resulting in a free flowing powder. The ProLease microspheres are administered in an aqueous solution *via* subcutaneous or intramuscular injection. The drug is then released from the microsphere by diffusion and biodegradation of the polymer.

2.1.5 The Ideal Polymer for Drug Delivery[1,5]

The ideal polymer for drug delivery should be:

a) Chemically and mechanically stable

b) Easy and cheap to prepare

c) Free of leachable impurities

d) Easily functionalised and capable of high drug loading

e) Biocompatible

f) Biodegradable → The polymer should be degraded into smaller fragments which can be excreted form the body.

→ The degradation products should be non toxic and should not show an inflammatory response.

→ Degradation should occur within a reasonable period of time as necessary by the application.

The perceived advantages of the use of sporopollenin as a support in drug delivery are outlined in Chapter 3, Section 3.5.4.

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3 SPOROPOLLENIN

3.1 INTRODUCTION

Pollen grains are the male reproductive structure of angiosperms (flowering plants) and plants classed as gymnosperms (for example, conifers).[1, 2] Spores are produced by non-seed bearing plants such as ferns and mosses as part of the process of reproduction.[3] Pollen and spores possess a protective double walled outer structure made up of at least two basic layers. The inner layer (intine) is made up of almost pure cellulose and the outer layer (exine) is formed form a substance known as sporopollenin. [1,4-6]

The earliest recorded observation of the exine was from John (1814) and Braconnot (1829).[4,6] Both researchers recognised the inertness of the exine compared with the rest of the pollen wall and Braconnot introduced the term "pollenin" to describe the exine. Zetzsche and Huggler (1928) introduced the expression "sporonin" to describe the resistant exine material from *Lycopodium clavatum* spores.[6] Finally the collective name of "Sporopollenin" was used to describe the resistant exine material of pollen grains and spores.[4] Zetzsche and Huggler [4,6,7] also observed that the cytoplasmic contents, eg. fats, genetic material and proteins, of *Lycopodium clavatum* spores could be removed by heating the solvent extracted spores in NaOH solution. This gave nitrogen free membranes which retained their original shape and consisted of two layers. The inner layer was stained blue with iodine in H_2SO_4 which is characteristic of cellulose when treated in this way. This inner layer could be removed to leave the exine by treatment over several days with either cuprammonium hydroxide, 40% HCl, 72% H_2SO_4 or 80% H_3PO_4. The resistant material left over by these treatments is considered to be the

chemically resistant sporopollenin material. Following acid hydrolysis and acetylation octa-acetylcellobiose, a characteristic hydrolysis product of cellulose, was isolated from the solution.

The resistance of sporopollenin to biological and non-oxidative chemical attack means that pollen and spore exines are preserved in peat and sediments as intact recognisable structures. The cytoplasmic, genetic and polysaccharide components are destroyed and only the very resistant exine remains.[8]

The study of pollen grains and spores is called Palynology and it has been useful in a wide range of fields such as forensic science, allergy studies and fossil and pollen analysis.[2]

Brooks and Shaw (1968) recovered a residue very similar to modern sporopollenin from the early sedimentary rocks of the Onverwacht (around 3.7 billion years old). [4,8-10] This has been important in tracing past vegetation history and the origins of life. Brooks and Shaw have also found that the insoluble matter found in some meteorites resembles modern sporopollenin.[8,11]

3.2 ISOLATION OF SPOROPOLLENIN FROM MODERN POLLEN AND SPORES

There have been numerous methods in the literature used to isolate sporopollenin from fresh pollen grains and spores. Some involve harsh treatments with strong acids and bases at elevated temperatures and other methods use gentle procedures to isolate sporopollenin. It has been suggested that some methods, in particular the harsh treatments may bring about changes to the sporopollenin structure[12,13] and this must be

taken into consideration when comparing sporopollenin samples prepared using different methods. The main methods are detailed as follows:

3.2.1 Treatment with Alkali and Phosphoric Acid

Briefly, this method involves treatment of the spores/pollen with organic solvents followed by a hot alkali treatment and then treatment with warm 80% orthophosphoric acid for 6 days. This method was first used by Zetzsche and Huggler (1928) [1,4,6] and was also used by a number of different workers.[14,15,16] Variations in the solvents used and of the length of time and temperature at the phosphoric acid stage have been reported.[12,17-20] More recently, Shaw *et al* (1996) found that phosphoric acid alone did not completely remove cellulose and an extra treatment with 80% H_2SO_4 was necessary to remove all traces of cellulose.[17]

3.2.2 Acetolysis

The acetolysis method for the extraction of sporopollenin was introduced by Erdtman (1960).[21] This involved treatment of the pollen/spores with a 9:1 mixture of acetic anhydride and concentrated H_2SO_4. Other researchers have used or adapted this acetolysis method.[16,22-26]

3.2.3 4-Methlmorpholine-*N*-Oxide

4-Methylmorpholine-*N*-oxide monohydrate (4-MMNO.H_2O) has been used for the effective separation of the sporopollenin exine from the remainder of the pollen or spore.[27-29] Pollen was suspended in molten 4-MMNO.H_2O at 75°C where rapid release of sporoplasts (pollen contents which have been stripped of its exine) occurred. Figure

3.1 shows scanning electron micrographs of sporoplast release in Lily pollen.[28] Aqueous solutions using 4-MMNO.H_2O have also been used at room temperature and 70°C.[27,28] Both sporoplasts and pure sporopollenin have been recovered using these treatments.

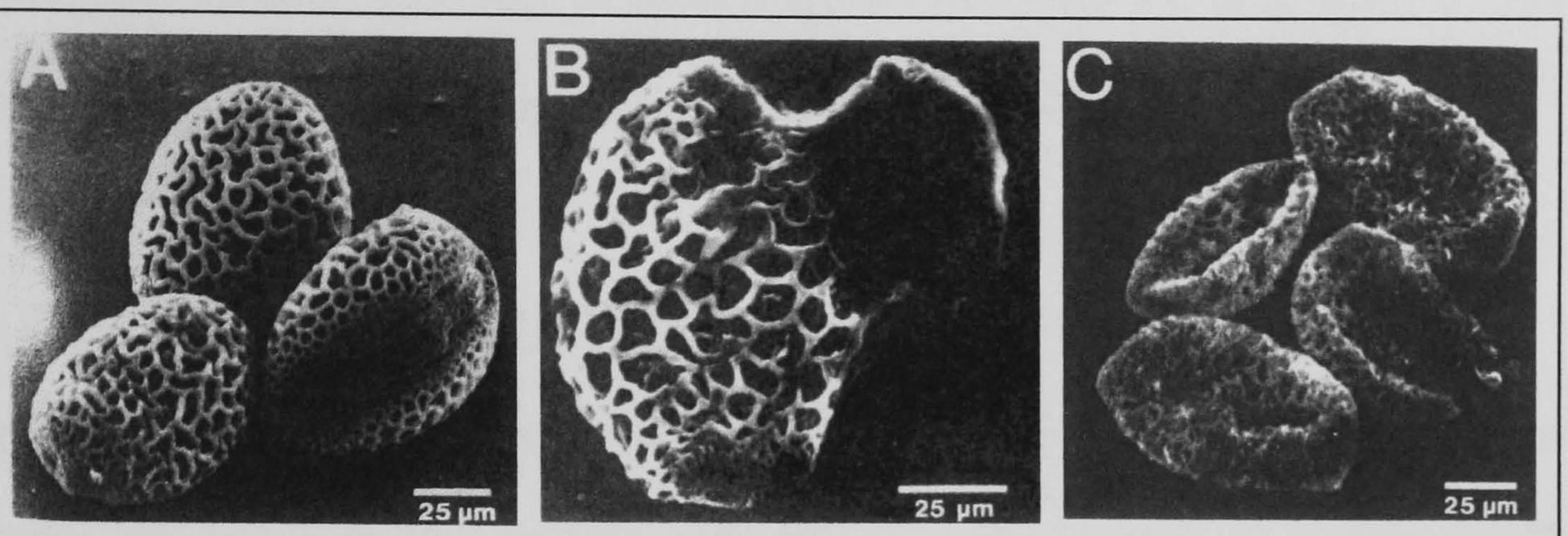


Figure 3.1 Scanning electron micrographs of A) Intact Lily pollen, B) Sporoplast release and C) Exines after sporopolast release.[29]

3.2.4 Anhydrous HF

Dominguez *et al* (1998) developed a method of isolating sporopollenin using anhydrous HF in pyridine at 40°C for 5 hours.[30]

3.2.5 Enzymes

A number of workers have used enzymes to recover sporopollenin or have incorporated an enzymatic hydrolysis step into their sporopollenin extraction procedure.[31-34]

These procedures are believed to produce sporopollenin which remains intact and retains the full morphology of the original pollen grain or spore.

3.3 STRUCTURE OF SPOROPOLLENIN

Because of the extreme resistance of sporopollenin to chemical attack the structure of sporopollenin has not yet been fully established. A review of the literature presented in this thesis has revealed that there is not one agreed mechanism of how sporopollenin is produced biochemically nor what its structural components are.

3.3.1 Work by Zetzsche *et al* (1928-1937) [1,4,6,35,36]

Experiments began on the examination of the membranes of pollen and spores an in particular the sporopollenin structure in 1928 by Zetzsche and co-workers.[6] They isolated sporopollenin from various pollen grains, spores and fossil material in order to identify their composition. A summary of this work is shown in Figure 3.2.

3.3.1.1 Elemental Analysis

Results of elemental analyses showed that sporopollenin exine (intine removed) is composed of C, H and O. The sporopollenins were represented by an empirical formula based on a C_{90} unit which varied from species to species. The elemental analyses of a number of sporopollenins are shown in Table 3.1.[1,6,36]

3.3.1.2 Functional Group Identification

a) Sporopollenin was shown to contain hydroxyl groups.[6,36] Acetylation with acetic anhydride followed by saponification with alkali gave values for the number of hydroxyl groups per C_{90} unit

b) Kuhn-Roth oxidations with chromic acid indicated the presence of C-Me groups.[36]

3.3.1.3 Reaction With Bromine

It was suggested by Zetsche *et al* that sporopollenin contains substantial unsaturation as treatment of sporopollenin from *Lycopodium clavatum* spores with bromine in CCl_4 produced bromosporopollenin with approximately 50% bromine content.[6]

3.3.1.4 Oxidative Degradation

Sporopollenin is susceptible to oxidative chemical attack and can be degraded into simple soluble substances by oxidising agents.[6,36] Zetzsche *et al* exposed *Lycopodium clavatum* membranes to ozone in acetic acid followed by treatment with hydrogen peroxide. This procedure dissolved most of the membrane wall and a mixture of simple dicarboxylic acids, especially malonic, succinic, glutaric, and adipic acid (Ratio of 1:2:1:1), were obtained.

SPOROPOLLENIN SOURCE	EMPIRICAL FORMULA	C-Me / Mole	OH / Mole
Lycopodium clavatum (spores)	$C_{90}H_{144}O_{27}$	2.04	15
Pinus sylvestris (pollen)	$C_{90}H_{144}O_2$	1.70	13
Phoenix dactylifora (pollen)	$C_{90}H_{150}O_{33}$	3.45	—
Tasmanian fossil pollen	$C_{90}H_{136}O_1$	3.00	—

Table 3.1 Results from analytical experiments carried out by Zetzsche *et al* (1937).[1,36]

The results from Zetzsche's experiments did not give any insight into the actual monomers present in the sporopollenin structure as all of the products formed from the degradation experiments were produced under harsh conditions which could have involved modification of the structural units.

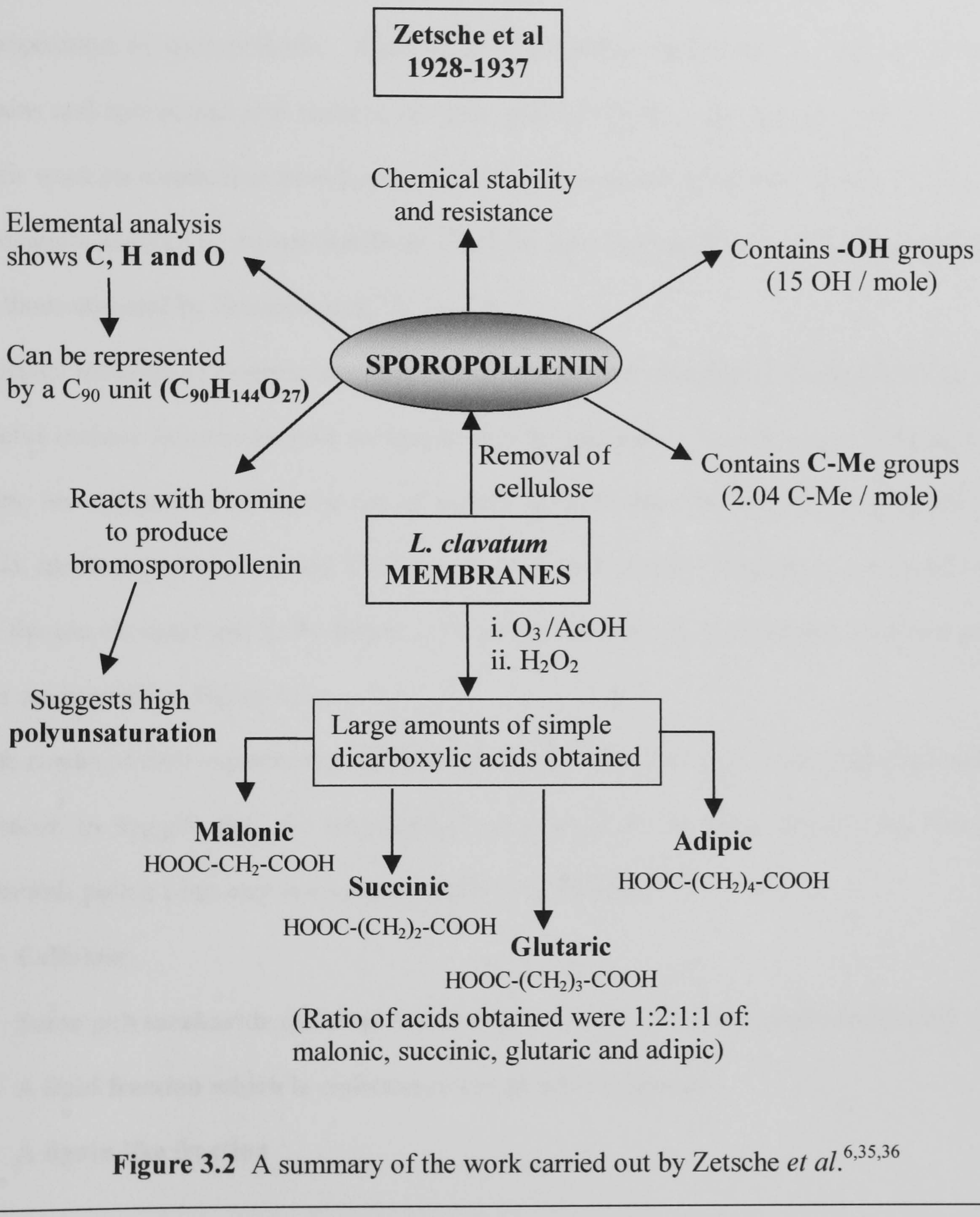


Figure 3.2 A summary of the work carried out by Zetsche *et al.*[6,35,36]

3.3.2 Early Work by Shaw and Yeadon (1964, 1966) [1,4,7,15]

Shaw and co-workers[1,4,7,15] reinvestigated the experiments carried out by Zetzsche *et al* and also carried out additional degradative studies in an attempt to determine the composition of sporopollenin. Shaw *et al*[7,15] prepared membranes of various pollen grains and spores and also isolated the sporopollenin exine. They carried out most of their work on membranes from *Lycopodium clavatum* spores and *Pinus Silvestris* pollen. Elemental analysis of the sporopollenin obtained from the membranes were very similar to those obtained by Zetzsche *et al*.[1,6]

Various methods of degradation were used in an attempt to degrade sporopollenin in a useful manner in order to gain an insight into its structure. A wide range of reagents were investigated including the use of sodium hypochlorite, chromic acid, strong nitric acid, treatment with ozone and KOH fusion. The most useful techniques were found to be the use of ozone and KOH fusion. The results obtained during treatment with ozone are summarised in Figure 3.3.

The results of their experiments and previous results from Zetzsche's work led Shaw and Yeadon to suggest that the membranes of *Lycopodium clavatum* spores and *Pinus silvestris* pollen were very similar and contain the following.[7,15]

1 Cellulose

2 Some polysaccharide material which is more readily hydrolysed than cellulose

3 A lipid fraction which is resistant to chemical treatments

4 A lignin like fraction

3.3.2.1 Cellulose

Treatment of the membranes of *Lycopodium clavatum* spores with a smaller amount of ozone[3,7,15] of that used by Zetzsche resulted in the formation of an insoluble solid (80-

90% by weight of the original membrane) and a solution from which an oil (30-35% by weight of the original membrane) was isolated. The solid mainly dissolved in sodium hydroxide at room temperature to leave an insoluble residue of cellulose (12% by weight of original membrane) which was confirmed by hydrolysis with H_2SO_4 to glucose and by IR comparison with a pure cellulose sample. The cellulose retained the shape of the original membrane.[15]

3.3.2.2 Additional Polysaccharide Material

Shaw *et al*[15] repeated Zetzsche's ozone degradation and also found that the majority of the membrane dissolved leaving a mixture of simple dicarboxylic acids and a small amount of an insoluble residue. Acid hydrolysis of the residue produced cellulose and a solution which contained a mixture of sugars including xylose as the main component, glucose and galactose. Shaw[1,15] suggested that this polysaccharide material present in the solution could possibly be a hemicellulose or a xylan like material.

3.3.2.3 Lipid Fraction

Oxidative degradation[1,15] (see Figure 3.3 for a brief summary) gave a mixture of non-branched mono and di-carboxylic acids. After treatment with ozone (method of Shaw)[15] the resulting oil gave positive tests tests for oxo and hydroxy acids and after reduction with $NaBH_4$ the mixture was shown to contain hexadecanedioic acid and a mixture of alkanoic acids with the major one being identified as methyl palmitate.

After acidification of the solution obtained from the treatment of the insoluble solid with NaOH at room temperature, crystalline, 6,11-dioxohexadecanedioic acid was obtained.

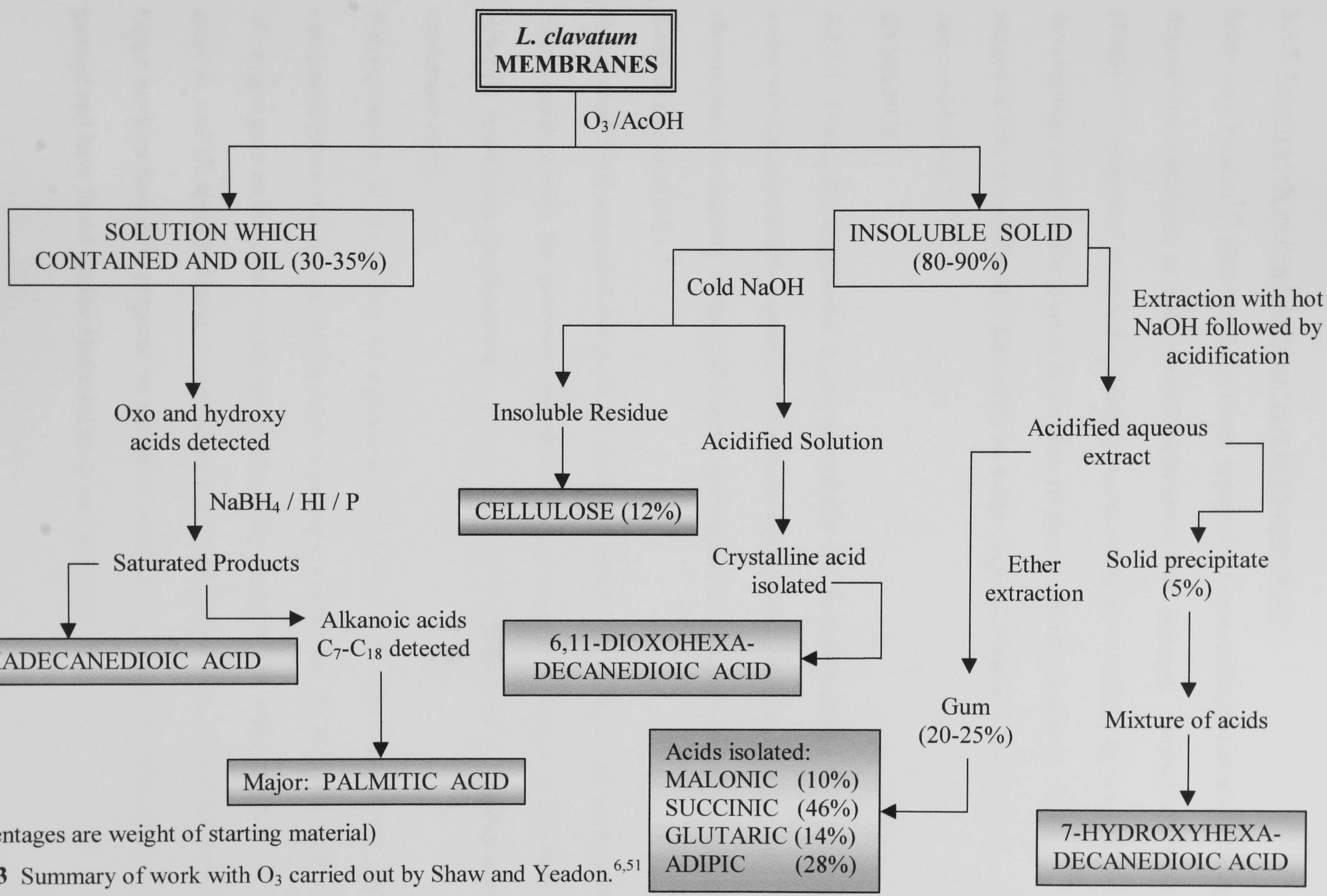
Acidification of the insoluble solid after extraction with hot sodium hydroxide gave a solid precipitate which contained a mixture of acids one of which was identified as 7-hydroxyhexadecanedioic acid. Ether extraction of the acidified extract gave a gum which after methylation and examination by GC consisted of a mixture of malonic (10%), succinic (46%), glutaric (14%) and adipic acid (28%). Shaw *et al*[1,15] believed that these acids could have been possible oxidative degradation products of the C_{16} acids identified earlier or oxidation products of similar like material of lipid origin.

3.3.2.4 Lignin Material

Fusion of the membranes of *L. clavatum* spores with KOH gave a mixture of alkanoic acids (C_5-C_{11}) and the phenolic acids p and m-hydroxybenzoic acid, protocatechuic acid and traces of syringic and vanillic acids. Shaw *et al* proposed that a lignin type fraction was present in the membrane as phenolic acids are obtained during a typical lignin degradation.[15] Infrared studies of the membranes showed absorptions around 1500-1600cm^{-1} which are characteristic of aromatic structures and similar bands have been found in lignins.[7,15,] However, methylation of the material with diazomethane followed by KOH fusion gave no methoxy aromatic acids as would be expected from a lignin type structure.[4] The membranes gave none of the usual tests for lignins but Shaw[15] suggested that it was because it was masked by the cellulose and lipid fractions.

Shaw *et al* suggested that the sporopollenin found by Zetzsche was composed of a lipid and a lignin type fraction.

The above experiments were carried out on the membranes of *P. Silvestris* pollen where similar results were obtained.[15]



(Percentages are weight of starting material)

Figure 3.3 Summary of work with O_3 carried out by Shaw and Yeadon.[6,51]

3.3.3 Carotenoids as Possible Precursors of Sporopollenin

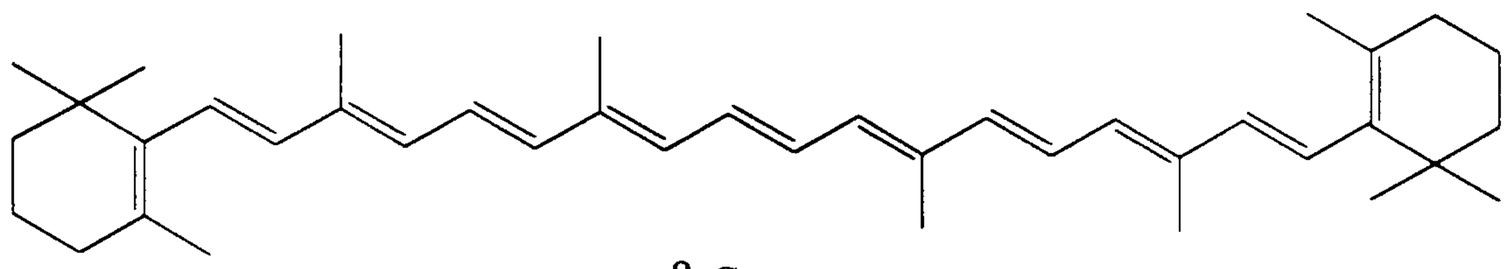
Shaw and Yeadon[7,15] abandoned the direct approach of sporopollenin identification by degradative techniques as only limited information was obtained. Brooks and Shaw (1968)[4,14,37] attempted to look for possible precursors of sporopollenin by studying the development of the pollen grain in *Lilium henryii* plants. Results from these studies suggested that sporopollenin is formed by an oxidative polymerisation of carotenoids and carotenoid esters.[14,37]

EVIDENCE:

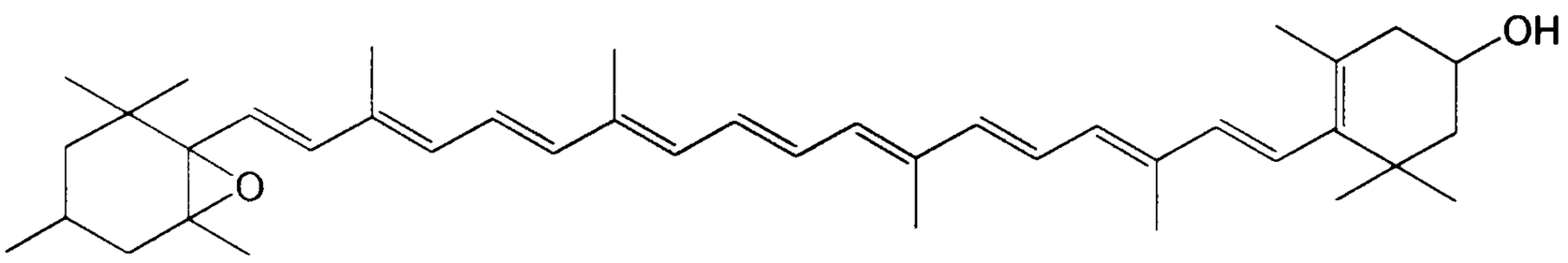
3.3.3.1 During the experiment it was noticed that the formation of the sporopollenin exine was accompanied by a parallel formation of carotenoids. Analysis of the extracts showed that the majority of the lipid formed consisted of free carotenoids and carotenoid esters in a ratio of 2.2:1.[4]

Brooks and Shaw suggested that the basic skeletal structure of carotenoid and carotenoid esters could provide the necessary chemical features required to produce a polymeric structure resembling sporopollenin.[1] Figure 3.4 shows a selection of carotenoids and carotenoid esters.

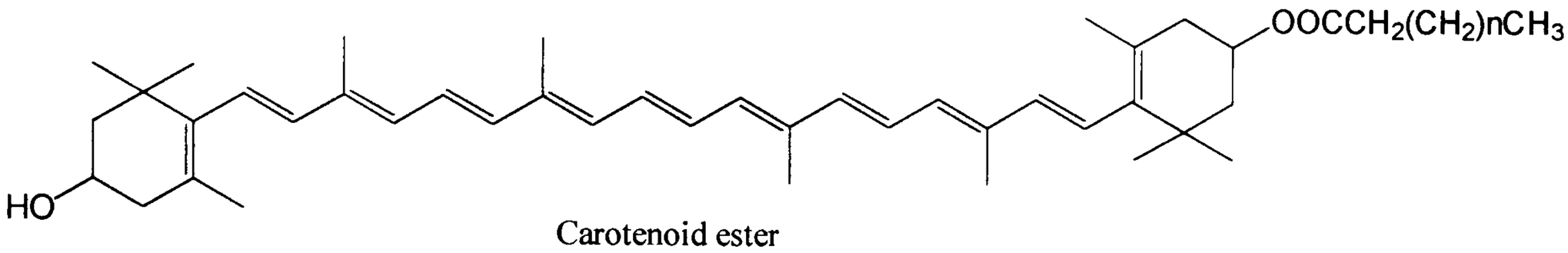
Polymerisation of a number of carotenoids including β-carotene, carotenoid and carotenoid esters of *L. Henryi* with a trace of a Lewis acid catalyst (BF_3) in the presence of oxygen gave an insoluble oxygen containing unsaturated polymer with elemental analysis and IR spectra similar to natural sporopollenin (See Figure 3.5 IR spectra).[14] Other workers have also prepared an oxidative polymer of β-carotene using the same method and have found similar elemental analyses (see Table 3.2).



β-Carotene



Antheroxanthin

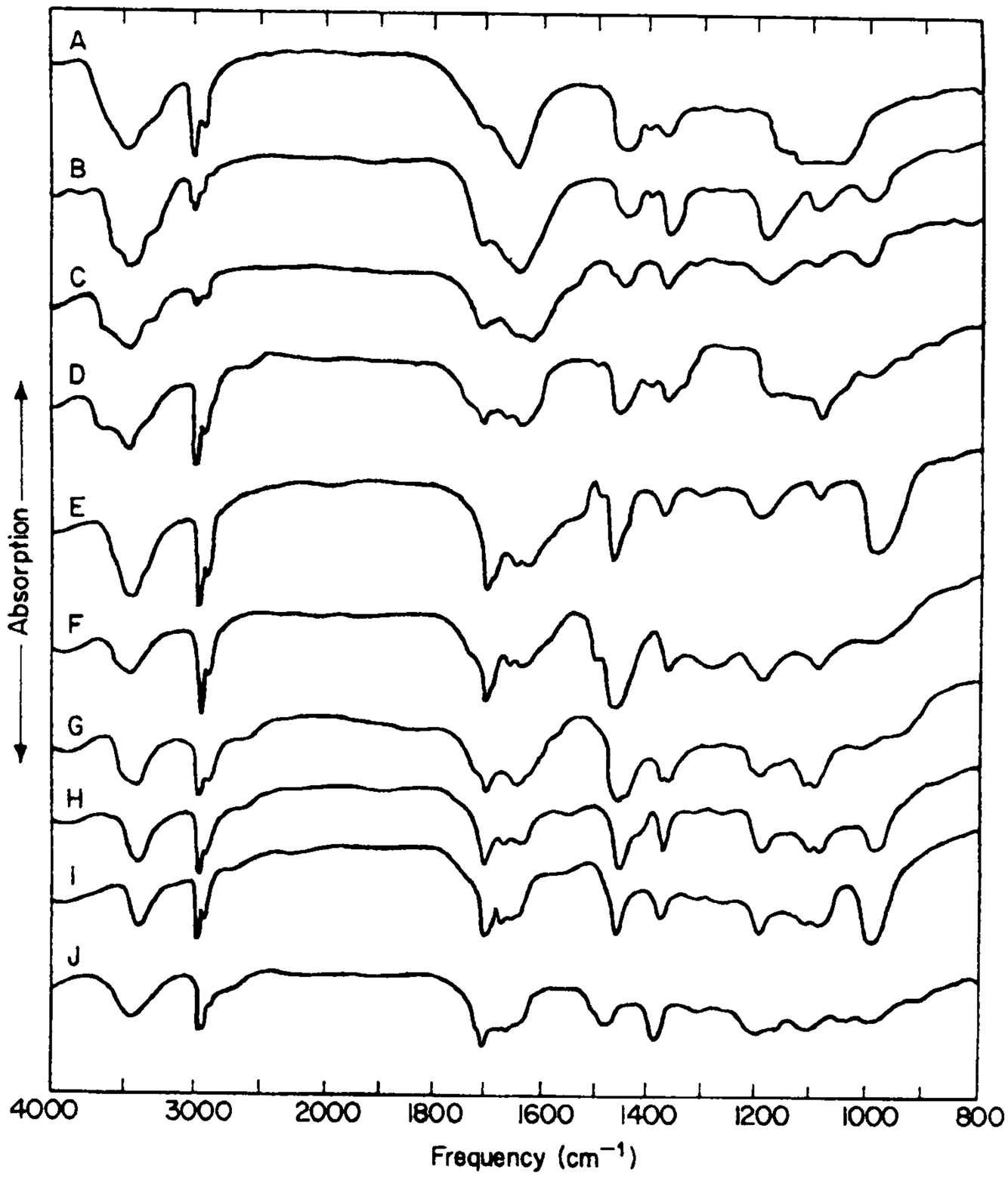


Carotenoid ester

Figure 3.4 Examples of Carotenoids and Carotenoid Esters.

SPOROPOLLENIN SOURCE	EMPIRICAL FORMULA	REFERENCE
Lycopodium clavatum spores	$C_{90}H_{144}O_{27}$ $C_{90}H_{138}O_{28}$	14 12
Lilium henryi pollen	$C_{90}H_{142}O_3$	14
Lilium henryi carotenoid polymer	$C_{90}H_{110}O_{33}$	14
Lilium henryi carotenoid ester polymer	$C_{90}H_{148}O_{38}$	14
Oxidative polymer of β-carotene	$C_{90}H_{130}O_{30}$ $C_{90}H_{119}O_{28}$ $C_{90}H_{132}O_{25}$	14 12 39

Table 3.2 Elemental composition of a number of natural and synthetic Sporopollenins. The C_{90} formula was used for comparative purposes.

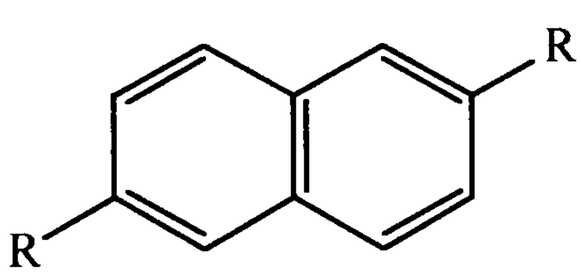


A. *Tasmanites huronensis* (Dawson)
B. Insoluble organic matter from the Orgueil Meteorite
C. Insoluble organic matter from the Murray Meteorite
D. Carboniferous megaspore (*Valvisisporites auritus*)
E. *Lycopodium clavatum* spore exine
F. *Lycopodium clavatum* spore exine after heat treatment
G. Polymerized β-carotene
H. *Pinus montana* pollen exine
I. *Lilium henryii* pollen exine
J. Synthetic polymer from *Lilium henryii* carotenoids and carotenoid esters

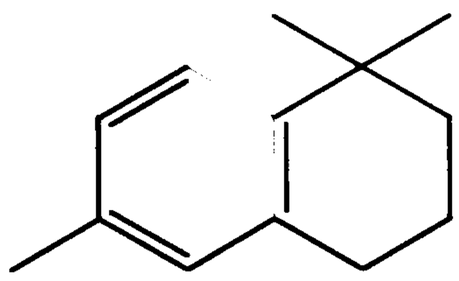
Figure 3.5 Infrared spectra of various sporopollenins[1]

The synthetic polymers were also resistant to acetolysis and insoluble in solvents and had similar degradation products when treated with ozone.[4] KOH fusion of the synthetic polymer and sporopollenin both obtained from *L. Henryi* pollen gave p-hydroxybenzoic acid as the major component. The fact that phenolic acids are produced from KOH fusion of polymerised carotenoids led Shaw and Yeadon to withdraw their original suggestion that sporopollenin is of lignin origin and Shaw more recently proposed that sporopollenin is produced by oxidative polymerisation of carotenoid esters.[14,37]

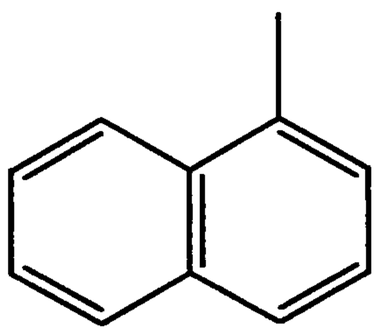
3.3.3.2 This proposal was further backed up by examining the pyrolysis products of sporopollenin from various sources such as modern pollen, coals and river shale. These products were compared with the pyrolysis products of β-carotene and its oxidative polymer and it was shown that the sporopollenin produced typical carotenoid degradation products.[38] β-Carotene, the β-carotene oxidative polymer and the sporopollenin material produced ionene and the most of the samples produced naphthalene, 2,6-dimethylnaphthalene and 1-methyl- and 2-methyl-naphthalene.[38] Figure 3.6 shows the structures of these products.



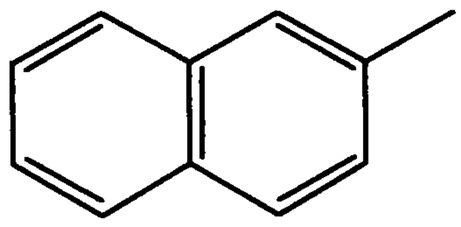
R = H (napthalene)
R = CH_3 (2,6-dimethyl napthalene)



ionene



1-methylnapthalene



2-methylnapthalene

Figure 3.6 Pyrolysis products from Sporopollenin and the β-carotene derived polymers.

3.3.3.3 Radiochemical experiments have also shown that ^{14}C labelled carotenoids were incorporated into the sporopollenin of various plants and fungal spores.[4]

3.3.3.4 Sporopollenin is also present in many algal and fungal spores and these spores also synthesise carotenoids. The spores of the fungus *Rhizopus sexualis* do not possess sporopollenin in their outer wall and do not produce carotenoids.[40]

3.3.4 Inhibition and Radiolabelling Studies on Sporopollenin Biosynthesis

3.3.4.1 Effects of inhibitors of carotenoid synthesis on sporopollenin

In contradiction to the findings by Shaw,[4,14,37] Prahl *et al* (1985)[16] demonstrated that a severe influence in the formation of carotenoids did not inhibit sporopollenin biosynthesis. Sandoz, a known inhibitor of carotenoid synthesis, was applied to the plants of the *Cucuribita* (pumpkin) and it was found to influence carotenoid metabolism at the site of sporopollenin synthesis by hindering the desaturation of carotenoids and therefore saturated precursors accumulated. Sporopollenin from the pollen was isolated and it was found that sporopollenin biosynthesis was only slightly affected. As Sandoz only affects the final stages of carotenoid synthesis it could not be ruled out that saturated carotenoids or precursors could take part in the sporopollenin biosynthesis. Additionally, traces of unsaturated carotenoids such as β-carotene and antheraxanthin are still produced and these traces could be present in sufficient amounts to ensure a normal sporopollenin synthesis. The authors[16] therefore concluded that an interruption of carotenoid synthesis did not disturb sporopollenin accumulation.

In addition to these studies, Heslop-Harrison *et al*[41] were unable to detect carotenoids during the stages of pollen ripening when sporopollenin undergoes an intensive accumulation.

3.3.4.2 Tracer experiments on sporopollenin biosynthesis

Gubatz *et al* (1993)[32] carried out tracer experiments with a number of radiolabelled substances as precursors on *Tulipa* and *Cucuribita* species. Phenylalanine was incorporated into the sporopollenin at a higher rate than any other precursor used. After KOH fusion of the sporopollenin labelled with ^{14}C-phenylalanine, p-hydroxybenzoic acid was the main labelled product among the ether soluble acids. It was suggested that it was unlikely that these acids formed a genuine structure of the sporopollenin skeleton as they were formed under forcing conditions. Gubatz *et al* instead proposed that a phenylpropane unit such a p-coumaric acid could be a monomer involved in the sporopollenin structure (see Figure 3.7).

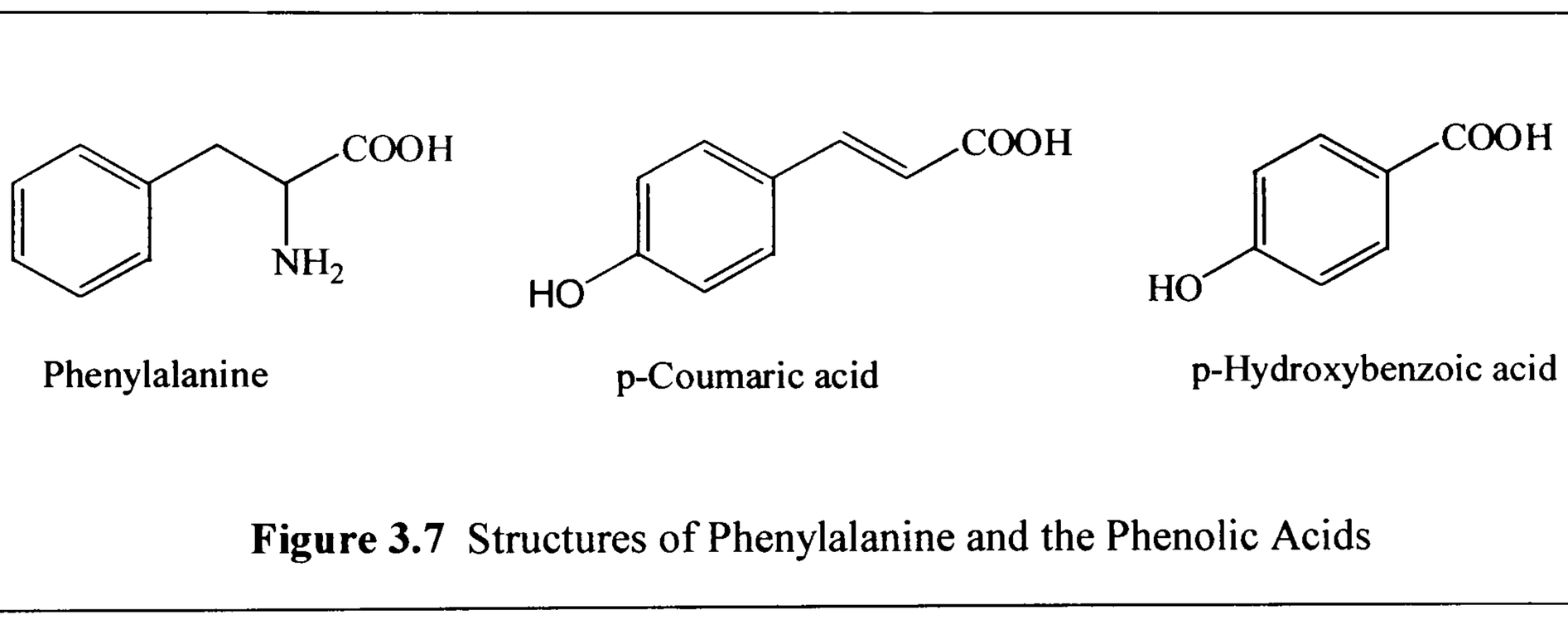


Figure 3.7 Structures of Phenylalanine and the Phenolic Acids

3.3.4.3 Incorporation of Oleic Acid into Sporopollenin [31]

Studies were undertaken on the green alga, *Scenedesmus actus*, whose outer cell wall is believed to be composed of sporopollenin. The incorporation of ^{14}C oleic acid into the green alga was inhibited by the chloroacetamide herbicide, Metazachlor. The sporopollenin was isolated after exposure of the green alga to ^{14}C oleic acid and it was found to be radioactive. After treatment of the cells with Metazachlor the incorporation of ^{14}C into the sporopollenin was inhibited by 98%. These results suggest that oleic acid,

which has the structure $CH_3(CH_2)_7$-CH_2=CH_2-$(CH_2)_7CO_2H$, is a precursor of sporopollenin and that the biosynthetic pathway is targeted by metazachlor.

3.3.4.4 Determination of the hydroxyl content of sporopollenin[39]

The number of hydroxyls present in a number of sporopollenins from various sources was determined by refluxing the sporopollenin with 1-C^{14}acetic anhydride. The incorporation of acetyl groups appeared to reach a maximum after 4 hours under refluxing conditions. The modern sporopollenins had hydroxyl values in the range 5.9-9.6 hydroxyls per C_{90} unit with sporopollenin from *L. clavatum* having 6.6 hydroxyls per C_{90} fragment. Synthetic polymers prepared from oxidative polymers of carotenoids and fossil sporopollenin had a lower number of hydroxyls which were between 1.7-1.9 hydroxyls per C_{90} unit.

3.3.5 Phenols as Integrated Components of Sporopollenin

It was mentioned earlier by Shaw and Yeadon[15] that phenolic acids were produced from potash fusion of *L. clavatum* and *L. Henryi* membranes. At first they suggested that sporopollenin was composed of a lignin type structure because of this observation but later withdrew their theory after the oxidative polymer of β-carotene produced phenolic acids after KOH fusion.[14] They believed that the phenolic acids were formed from the carotenoid skeletal structure under the forcing conditions during fusion with KOH.

Schulze-Osthoff and Wiermann (1987) carried out investigations on sporopollenin isolated from pine pollen and found that large amounts of phenols were obtained after nitrobenzene oxidation with p-coumaric acid being the main product.[33] Following fusion with KOH, p-hydroxybenzoic acid was predominant in the mixture of phenolic

compounds obtained. The large amounts of phenolic acids produced suggested that phenols are integral parts of the sporopollenin. The main products obtained after nitrobenzene oxidation possessed a phenylpropane skeleton and it was suggested that the hydroxybenzoic acids obtained after KOH fusion were degradation products of these phenylpropane units. The proposed involvement of phenylpropane units in the structure of sporopollenin was in accordance with the suggestions mentioned earlier by Gubatz *et al*[32] It was also noted that nitrobenzene oxidation of sporopollenin did not give characteristic degradation products of lignin and this was further evidence that sporopollenin differs in structure to lignin.[33]

In addition to these studies, Wehling *et al* (1989) analysed the sporopollenin of pine pollen by pyrolysis mass spectrometry and found that peaks indicative of p-coumaric acid were predominant in the spectrum.[42] The sporopollenin was also subjected to degradation using AlI_3 and p-coumaric acid was the major product released. AlI_3 is a gentle degradative method and is noted to be capable of splitting ether bonds. Because the treatment with AlI_3 is a very mild method compared to KOH fusion or nitrobenzene oxidation, Wehling *et al* suggested that this was evidence to prove that p-coumaric acid is a genuine component of sporopollenin which may be bound by ether linkages. The oxidative polymer of β-carotene was treated with AlI_3 and no p-coumaric acid or any other phenolic products were detected. This suggested that the β-carotene polymer differed structurally to the natural sporopollenin.

3.3.6 The Use of Spectroscopy in the Analysis of Sporopollenin

3.3.6.1 NMR Spectroscopy

^{13}C Solid State NMR. This has been used to study sporopollenin from both modern and fossil sources. The important information from these studies are summarised in Table 3.3.

The main points of the research include:

a) The ^{13}C-NMR spectra of modern sporopollenin are similar with the spectra showing that the sporopollenins consist of unsaturated, aliphatic, oxygenated and aromatic carbon.

b) There are slight differences in the spectra of the major plant groups such as higher plants, mosses and alga.

c) Sporopollenin may be derived from fatty acid precursors.

d) Fossil sporopollenin spectra are different to modern sporopollenin spectra in that the fossil sporopollenins seem to have lost oxygen containing groups possibly due to their age and the processes which occur during fossilisation.

It was observed that different workers obtained slightly different ^{13}C-NMR spectra of sporopollenin from the same species, for example, most of the researchers studied the spectra of *Lycopodium* spores and when these spectra were compared it was evident that there were some variation. This was most likely due to the various methods of isolation used by different groups of workers. Evidence for this was demonstrated by Hemsley *et al*,[22,24,26] who used acetolysis to isolate sporopollenin and found the spectra showed the presence of acetyl groups which were introduced during the procedure. Shaw *et al* also showed that some methods of isolating sporopollenin left varying amounts of cellulose behind which again would leave sporopollenin with slightly different compositions.

This meant that only sporopollenin isolated using the same method could be properly compared in these studies. When taking into consideration the peaks in the ^{13}C-NMR spectra due to polysaccharide and acetyl groups the spectra obtained in the NMR studies were almost identical.

^{1}H-NMR. The ^{1}H-NMR spectra of sporopollenins dissolved in 2-aminoethamol have been recorded.[34] ^{1}H-NMR and 2D ^{1}H-^{1}H-COSY NMR spectra of sporopollenin from *Typha angustifolia* (angiospermae) and *Torreya californica* (gymnospermae) showed a high degree of similarity which suggested that the molecular structure of these two sporopollenins are similar. The spectra showed the presence of 1,4-disubstituted or 1,2,3,4-tetrasubstituted benzene systems which possessed various oxygen containing functionalities such as hydroxyls, carboxylic acids and esters.

Reference	Isolation Method	Information Gained
Guilford[20] (1988)	Use of organic solvents, KOH and H_3PO_4	→ The Sporopollenin of higher plants, moss and algae are similar in composition but small differences are evident between the different plant groups. → Four distinct regions of the ^{13}C-NMR spectra indicate the presence of unsaturated, aliphatic, carbonyl and oxygen containing carbon. → Suggested that sporopollenin is composed of fatty acid precursors.
Espelie [28] (1989)	MMNO.H_2O	→ Similar spectra to Guilford but differences noted. → Results indicate that sporopollenin is mainly aliphatic with large amounts of acid and ester groups. → A small amount of alkenic and/or aromatic carbon is present. → Possible presence of aromatic groups.
Hemsley[22,24,26] (1992, 1993, 1996)	Acetolysis	→ ^{13}C-NMR spectra of modern sporopollenin are similar but slight variations are observed between plant groups. → All sporopollenins show unsaturated, aliphatic, aromatic and oxygenated groups. The proportions of these vary between species. → Possible presence of acids and esters. → Spectra of sporopollenin from Pinus and Lycopodium obtained are different to spectra produced by other workers. →The acetolysis introduced acetyl groups into sporopollenin.
Shaw and Apperley [17] (1996)	Use of organic solvents, KOH, H_3PO_4 and H_2SO_4	→ Sporopollenin isolated using H_3PO_4 was different to the spectra isolated using H_3PO_4 followed by H_2SO_4. → It became evident that H_3PO_4 alone appeared not to remove all the cellulose and an extra step with H_2SO_4 was required to remove the cellulose material. → The spectra of sporopollenin from Lycopodium spores treated with H_2SO_4 was very similar to the spectra of the oxidative polymer of β-carotene.

Table 3.3 Information obtained form ^{13}C-NMR spectra of sporopollenin.

3.3.6.2 IR Spectroscopy

Shaw *et al*[1,15] demonstrated that the infrared spectra of sporopollenin from modern and fossil sources are similar. The oxidative polymer of β-carotene also has a similar infrared spectrum (See Figure 3.5).[1,15] Shaw and Yeadon[51] measured the IR spectra of a number of membranes including spores from *Lycopodium clavatum*, pollen from pine oak and willow and pollen from a number of various grasses. The results from these studies are summarised in Table 3.4.

Region (cm^{-1})	Information Gained
4000-2000	Absorptions present near: → 3400 cm^{-1} (strong, broad) indicates the presence of hydroxyl groups. After reaction with acetic anyhdride the intensity of the peak is reduced. → 2930 cm^{-1}(strong, sharp) and 2860 cm^{-1} (strong, sharp but less intense) are probably due to CH_2 stretching frequencies.
1710-1640	→ All showed broad peak in this region indicating the presence of C=O containing groups. → No absorption at around 1740 cm^{-1}. Compounds which contain ester groups usually absorb around this region. Evidence for the absence of ester groups. *L. clavatum* membranes after methylation with diazomethane showed no absorption at 1740 cm^{-1} which suggests the lack of carboxyl groups.
1600-1500	→ All membranes showed absorption bands in this region. The peaks around 1510 cm^{-1} showed an increase in intensity after diazomethane treatment indicating the presence of phenolic groups.
1500-700	→ A broad absorption near 1100 cm^{-1} may be due to ether groups.

Table 3.4 Information from infrared spectra of some sporopollenins.[15]

Other workers have recorded IR spectra of sporopollenins and have found similar results to that of Shaw *et al.* Kawase *et al*[23] also showed from IR studies that sporopollenin contained hydroxyl groups, ethers, C=O groups, possibly of the structure R-CO where R= aromatic, aliphatic or conjugated group. Kawase *et al* suggested that the main structure of sporopollenin is a simple aliphatic polymer which had aromatic and conjugated side groups. From these studies sporopollenin seems to consist of a main structure with side chains that vary between species.

3.3.6.3 X-Ray Photoelectron Spectroscopy (XPS)[23]

XPS was carried out on sporopollenin obtained from *Magnolia grandiflora* and *Hibiscus syriacus* pollen. This showed that C, H and O was present and that the basic structure of sporopollenin was nearly the same except for small variations between the two species. The results suggested that there are main structural features present in all sporopollenin but different side chains may occur.

3.3.6.4 UV-VIS Spectroscopy

The UV-visible spectra of sporopollenin from *M. grandiflora* and *H. syriacus* dissolved in 2-aminoethanol was also produced by Kawase *et al.*[23] The spectra showed the existence of aromatic groups and groups with conjugated side chains. The UV-visible spectra detected small structural variations which were present between the two plant groups.

3.4 FUNCTIONALISATION OF SPOROPOLLENIN

Sporopollenin after being suitably functionalised has been used as a support for peptide synthesis[18,43] and its use as an ion- and ligand exchange material has also been investigated.[44,45]

3.4.1 The Use of Sporopollenin in Peptide Synthesis

Preliminary experiments by Mackenzie and Shaw (1980)[43] showed that:

Sporopollenin from *Lycopodium clavatum* could be chloromethylated using stannic chloride and chlorodimethyl ether to give a chloride loading of around 1 mmol/g which was removable with pyridine.

1 Amino acids could be attached to the chloromethylated sporopollenin and then removed using HBr in TFA.

2 Simple tripeptides were prepared using chloromethylated sporopollenin which could be then cleaved and isolated.

3 After these reactions the sporopollenin morphology was unaltered.

The only disadvantage of this method of functionalisation was that the reagents used for chloromethylation were highly toxic and carcinogenic.

Adamson et al. (1983)[18] prepared a tetrapeptide on functionalised sporopollenin also obtained from *Lycopodium clavatum*. Figure 3.8 outlines the strategy used for the synthesis of the tetrapeptide using sporopollenin. Amination of sporopollenin with 1,3-diaminopropane resulted in a loading of 1.2 mmol/g of base (determined using the picric acid assay) after refluxing for 16 hours and a maximum loading of 1.6 mmol/g of base after 24 hours.

Adamson et al found that the NH group attached to sporopollenin was either only weakly basic (nucleophilic) or inaccessible. Evidence to support this statement came from the results of the following experiments:[18]

3.4.1.1 Sporopollenin aminated with triethoxy aminopropyl silane gave a loading of 0.8 mmol/g of base after assay with picric acid but no attachment of acetic anhydride, succinic anhydride or Fmoc alanine hydride resulted. The amount of base found after the reaction remained the same. This indicated that the amino end of the amine had reacted with sporopollenin.

3.4.1.2 When sporopollenin aminated with 1,3-diaminopropane was reacted with either acetic anhydride or chloroacetic anhydride, the amount of base was found to be halved according to the picric acid assay.

3.4.1.3 Treatment of sporopollenin aminated with 1,3-diaminopropane or triethoxy aminopropylsilane with acetyl chloride showed that no basic groups were present according to the picric acid assay. This implied that both ends of the attached diamine had reacted with the acid.

3.4.1.4 Sporopollenin after amination with 1,3-diaminopropane followed by reaction with either chloroacetic anhydride or chloroacetyl chloride, was reacted with the sodium salt of the 4-hydroxybenzyl alcohol linker to give a loading of 0.8 mmol/g, determined by chloride displacement. The picric acid assay showed that after attachment of the linker, 0.8 mmol/g of base was left. This meant that only the outer NH-chloroacetyl group had reacted and the inner NH-chloroacetyl group had been removed.

These results suggested that the NH end of the diamine attached to sporopollenin was not as reactive as the free amine end of the diamine.

The sporopollenin with the attached 4-hydroxybenzyl alcohol linker was then used for the preparation of the tetrapeptide which was built up by the addition of individual Fmoc-amino acid anhydrides. After each addition the sporopollenin was reacted with acetic anhydride to cap any unreacted hydroxyls. After addition of the last amino acid, the final loading of the tetrapeptide was found to be 0.23 mmol/g. (19% yield based on initial loading of linker)

3.4.2 The Use of Sporopollenin as an Ion and Ligand Exchange Medium

Functionalised sporopollenin from *L. clavatum* spores has been used for the successful separation of various nucleosides, nucleotides, α-amino acids and transition metals.[44,45]

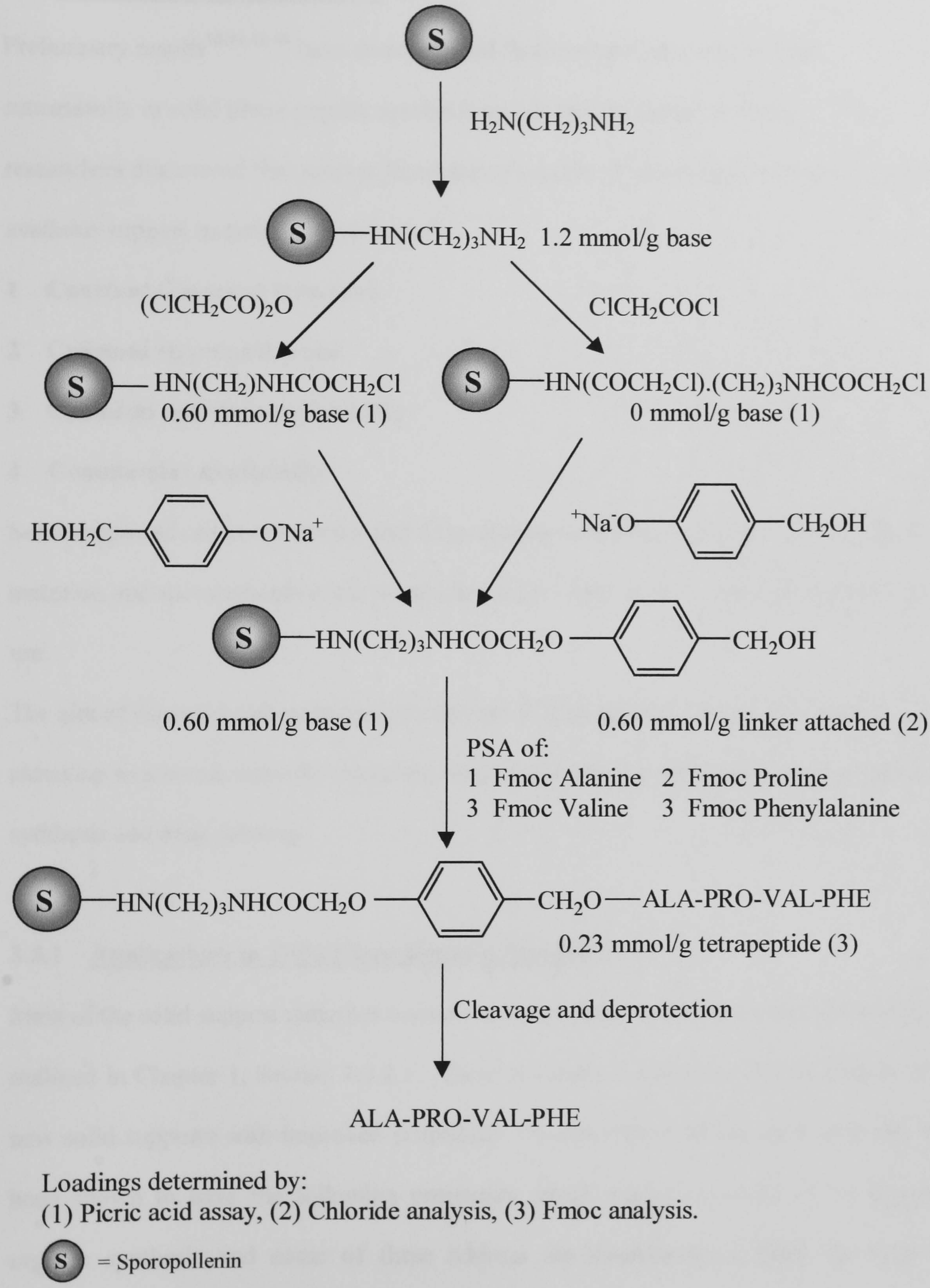
Sporopollenin was functionalised with various reagents which are shown in table 3.5

Reagent	Sporopollenin derivative (S = Sporopollenin)	Medium	Compounds Separated	Loading (mmol/g)
1,2-diaminoethane	S—$NHCH_2CH_2NH_2$	Anion exchanger	Ribonucleotides, Transition metals	1.59 (1)
Chlorosulphonic acid	S—SO_3H	Cation exchanger	α-amino acids	1.60 (2)
Bromoacetate and $CuCl_2$	S—$NHCH_2CH_2N(CH_2CO_2)_2^{2-}Cu^{2+}$	Ligand exchanger	Ribonucleosides	1.40 (3)

Loadings determined by (1) Picric acid assay, (2) Titrimetric analysis, (3) Method of analysis not given.

Table 3.5 Functionalised sporopollenins used in ion and ligand exchange.

Metal ligand complexes based on aminated sporopollenin from *L. clavatum* have also been used for the sorption of heavy metal ions from aqueous solution.[46] These have potential applications in the treatment of water contaminated with heavy metals.



Loadings determined by:
(1) Picric acid assay, (2) Chloride analysis, (3) Fmoc analysis.

S = Sporopollenin

Figure 3.8. The use of functionalised sporopollenin in peptide synthesis.

3.5 AIMS OF THE PROJECT

Preliminary results[18,43,45,46] have demonstrated that sporopollenin can be used successfully in solid phase peptide synthesis and in ion and ligand exchange. These researchers discovered that sporopollenin had a number of advantages over commercially available support materials which include:

1 Constant Chemical Structure

2 Constant spore/pollen size

3 Chemical and Physical Stability

4 Commercial Availability

Solid supported organic synthesis and drug delivery also rely on the use of solid support materials and sporopollenin could be an alternative to the support materials currently in use.

The aim of this work was to investigate the use of sporopollenin from *Lycopodium clavatum* as a novel, naturally occurring support material for use in solid phase organic synthesis and drug delivery.

3.5.1 Applications in Solid Phase Organic Synthesis

Most of the solid support materials commercially available suffer from the disadvantages outlined in Chapter 1, Section 1.3.2.1. There is clearly the need for the development of new solid supports with improved properties. As mentioned earlier, sporopollenin has been shown to have the following properties which would be useful in solid phase organic synthesis and some of these address the disadvantages faced by supports currently in use.

3.5.1.1 Constant Chemical Structure

It was believed[43,45] that the precise and constant morphology of pollen and spores would suggest that the molecular structure of sporopollenin would be homogeneous and non variable from grain to grain.

This would mean that sporopollenin prepared on separate occasions and samples of sporopollenin prepared or used by different workers would be the same. Additionally, after functionalisation of the sporopollenin, reproducible loadings should be observed between batches. This would be advantageous as new methods of preparing polystyrene supports have been developed which result in only a slight batch variation in functionality.[47,48] However, the majority of solid support materials still have loadings which vary between batches,[48,49] for example, Novabiochem (CN biosciences) offer Wang resin with a loading anywhere between 0.5-1.3 mmol/g.[48]

It has been noted from earlier studies[12,13] (and Section 3.3.6) that sporopollenin isolated using different methods vary slightly in their composition so it would be essential that sporopollenin to be used in solid phase organic synthesis is prepared using the same method each time.

3.5.1.2 Constant Spore/Pollen Size

Sporopollenin from *Lycopodium clavatum* (commonly known as Club Moss) has been reported to have an average diameter of around 20μm which is constant for each grain.[45] Columns of this material were free flowing and the flow rate was unaltered over a period of use of six months.[45] The advantage of using sporopollenin would be that the size of each spore or pollen exine would be the same unlike the variation in particle size seen within batches of other support materials used.[48,49]

Other pollen and spores could be also be used and sizes of these vary from 5μm to 250μm[1,5] which would provide a wide range of particle sizes. Only very small differences in size have been observed within the same species (for example, *Lycopodium selago,* a close relative of *Lycopodium clavatum*, have diameters between 28-33 μm and an average diameter of 30.5μm) and this is most likely to be influenced by external factors such as temperature, mineral nutrition and water conditions during growth and development.[5]

However IR and NMR studies of sporopollenin from different sources for example, different plants and mosses, (see Section 3.3.6) have been shown to differ in their structural composition. This would have to be taken into account if sporopollenin from various plant sources was to be used.

3.5.1.3 Chemical and Physical Stability

As mentioned earlier, the stability of sporopollenin is demonstrated by the survival of pollen and spore exines in ancient sedimentary rocks.[4,8-10]

Sporopollenin is stable to organic and aqueous solvents and most chemical reagents such as hot alkali and strong acid but is degraded by prolonged exposure to oxidising agents such as ozone.[1,4] Strong acids such as 80-85% H_3PO_4 and a mixture of acetic anhydride and concentrated H_2SO_4 have been used to isolate sporopollenin from many modern spores and pollen grains. Concentrated HF and HCl have been used to isolate fossil sporopollenin from sedimentary rocks.[1,2]

Many pollen grains and spores have been dissolved in 2-aminoethanol but sporopollenin from *L. clavatum* and many others were resistant to this treatment.[50] A wide range of pollen and spore exines have been shown to dissolve in 4-MMNO.H_2O.[27-29]

The susceptibility to oxidation is also demonstrated by corrosion of pollen and spores in aerated peats, conditions with minimal oxygen are thought to maintain maximum preservation.[1] Havinga *et al* (1984) undertook a 20 year investigation into the corrosion susceptibility of 19 different species of pollen and spores in various soil types.[51] The soil types were classed as having low or high biological activity. In soils with low biological activity and low pH, the deterioration of the pollen/spores was slow. Decay was rapid in soils with high biological activity and higher pH. From all the spores and pollen grains tested, *L. clavatum* spores were the most resistant and only 4% of the spores were found to be poorly preserved in soil with a high biological activity and high pH.

The stability of the sporopollenin after functionalisation and subsequent use in peptide synthesis and ion and ligand exchange was very good. The sporopollenin remained unchanged and retained the morphology of the original spore.[45] Columns of the material were very free flowing and no fragmentation into smaller particles was observed.[43,45]

It has also been reported that sporopollenin is stable to temperatures up to at least 250°C.[4,45]

Sporopollenin could make an alternative support to the resins which cannot withstand harsh reaction conditions and which breakdown easily.

3.5.1.4 Commercial Availability

Lycopodium clavatum spores are commercially available from a number of sources and it is not expensive. Around 20% of the *Lycopodium* spore membrane is composed of sporopollenin[1,4] so it can be obtained cheaply and in quantity from this spore source. The preparation of sporopollenin is also convenient and easy.

3.5.1.5 Functionalisation

Sporopollenin from *Lycopodium clavatum* has been functionalised for applications in ion exchange and solid phase peptide synthesis[18,43] in a number of ways:

a) Chloromethylation
b) Amination

The loadings of these functionalised sporopollenins fall within the ranges of commercially available functionalised resins used in solid phase organic synthesis.[47-48] Sporopollenin is easily functionalised using simple chemistry and cheap reagents.

3.5.2 Limitations of the Previous Work on the Functionalisation of Sporopollenin

Functionalised sporopollenin has been used as a support for peptide synthesis[18,43] and as an ion and ligand exchange medium[44-46] (See Section 3.4).

There have been a number of problems and drawbacks encountered with this earlier work which include the following:

1 There were no stability studies carried out to determine how stable the linkages were which attached the functional groups to sporopollenin.

2 There has been no previous work to examine the filterability and stability of sporopollenin in various solvents.

3 There were no explanations given or investigations undertaken into the type of attachments which resulted after functionalisation with the various reagents.

4 It was not clear how reproducible the loadings of functional groups attached to the sporopollenin were or how many times the functionalisation reactions were carried out.

5 The overall yield of the tetrapaptide prepared by Adamson *et al*[20] using sporopollenin was very low. No optimisation studies or further work has been reported in order to increase the yields.

6 There have been no attempts to investigate the use of the naturally occurring hydroxyls of sporopollenin as points of further attachment or modification.

7 No attempts to determine if there were are any other naturally occurring functionalities present on the sporopollenin surface which could be used or if these functional groups would interfere with any further synthesis.

3.5.3 Aims and Objectives for the Application of Sporopollenin as a Solid Support

Applications of sporopollenin in the areas of solid phase organic synthesis and in the use of immobilised reagents and scavenger resins in solution phase chemistry will be investigated. For sporopollenin to make a good solid support material the following criteria must apply:

1 Sporopollenin needs to be appropriately derivatised by direct functionalisation or by attachment of a linker.

2 The loadings of these functional groups need to be comparable with the loadings of commercial resins.

3 The functional groups should form stable attachments to sporopollenin.

4 These functional groups should be reactive towards further modification.

In order to assess the suitability of sporopollenin as a support, there is a need to explore the reactivity of sporopollenin and this will involve:

a) Reacting sporopollenin with a range of reagents to see which functional groups can be attached. The chloromethylation and amination reactions will be investigated

further along with the derivatisation of the naturally occurring functional groups, for example the hydroxyls, to see if they could be used as points of attachment for linking groups or used directly to attach building blocks.

b) The stability of these functional group attachments and linkers to a wide range of conditions frequently encountered in solid phase organic synthesis need to be examined.

c) Experiments will be undertaken to see if it is possible to determine the nature of attachments of the functional groups to sporopollenin.

d) The loadings of these functional groups attached to sporopollenin need to be properly assessed and checked for reproducibility and consistency.

Proposed Synthesis using Sporopollenin

To demonstrate the usefulness of sporopollenin as a solid support for solid phase organic synthesis, a series of reactions have been chosen which have not yet been carried out on solid phase because of the harsh conditions required to achieve the final product (See Figure 3.9). In the proposed work it is intended to attach an amino acid to sporopollenin via an ester linkage. This will be useful in comparing the reactivity and loadings with commercial material. The free amino group will then be reacted with the imidate **(A)** to give a 1-substituted-5-aminoimidazole **(B)**. This step uses mild conditions and will give an indication of the reactivity of the substituted sporopollenin support taking into account factors such as steric hindrance. The imidazole **(B)** attached to sporopollenin could be either cleaved at this stage to give the aminoimidazole **(C)** or cyclised to produce the purine **(D)**, which will give an indication of the robustness and stability of

the support and linking groups to harsh reaction conditions. This chemistry has already been carried out in solution.[52]

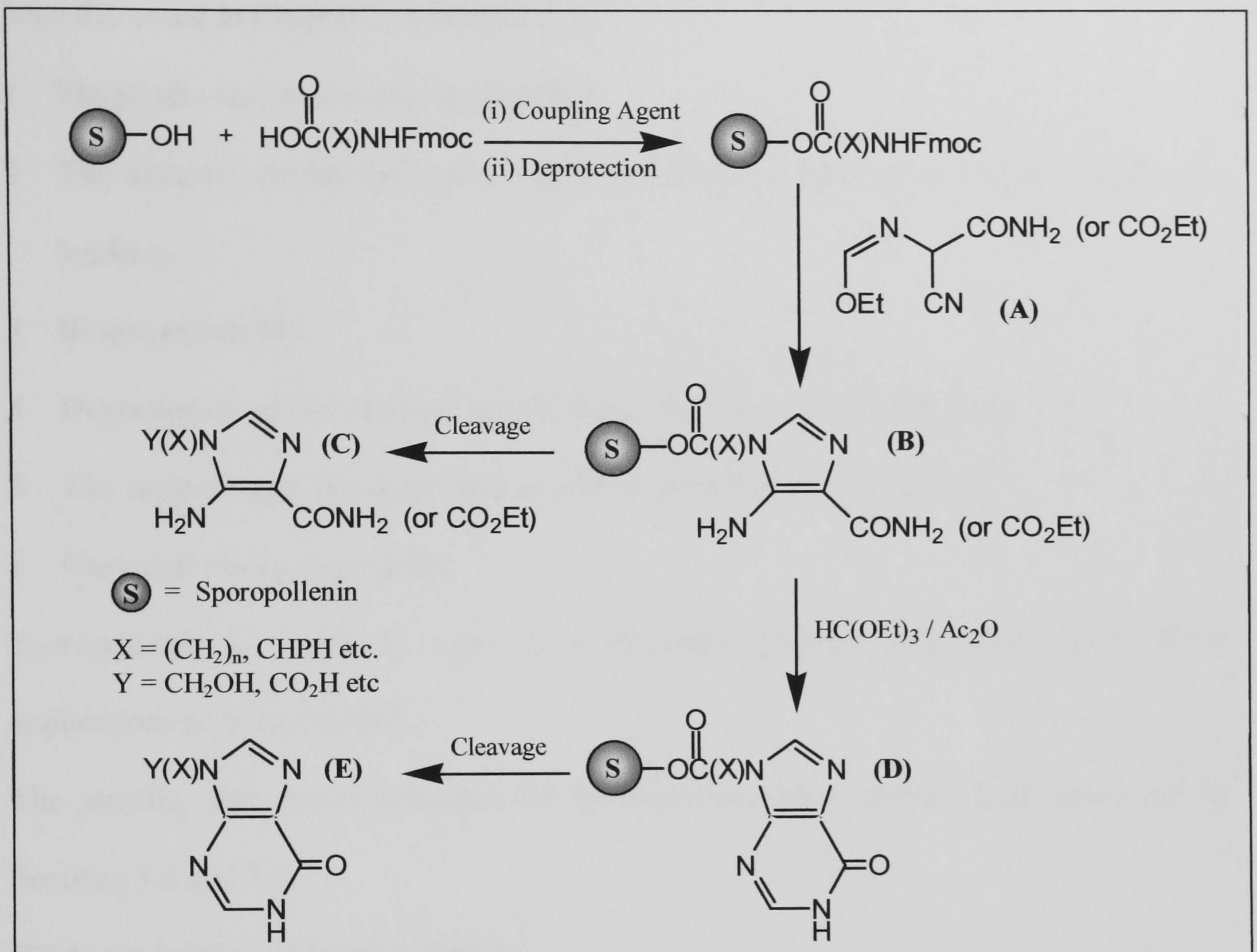


Figure 3.9 The synthesis of imidazoles and purines on sporopollenin.

To determine to usefulness of sporopollenin as a support for scavengers it will be necessary to attach these agents to sporopollenin and then expose them to conditions frequently encountered in solution phase purification using immobilised scavengers. Their performance can then be compared with commercially available scavenger resins.

3.5.4 Applications in Drug Delivery

The ideal solid support for drug delivery has the following properties which have already been discussed in Chapter 2, Section 2.1.5.

1 **Physically and mechanically stability.**

2 **The support should be easily functionalised and have a sufficiently high drug loading.**

3 **Biodegradability.**

4 **Degradation of the support should occur within a reasonable time.**

5 **The support and the degradation products should be non-toxic.**

6 **Easy and cheap to prepare**

Sporopollenin is found to have these essential qualities and could have future applications in drug delivery.

The stability and functionalisation of sporopollenin have already been discussed in Sections 3.4 and 3.5.

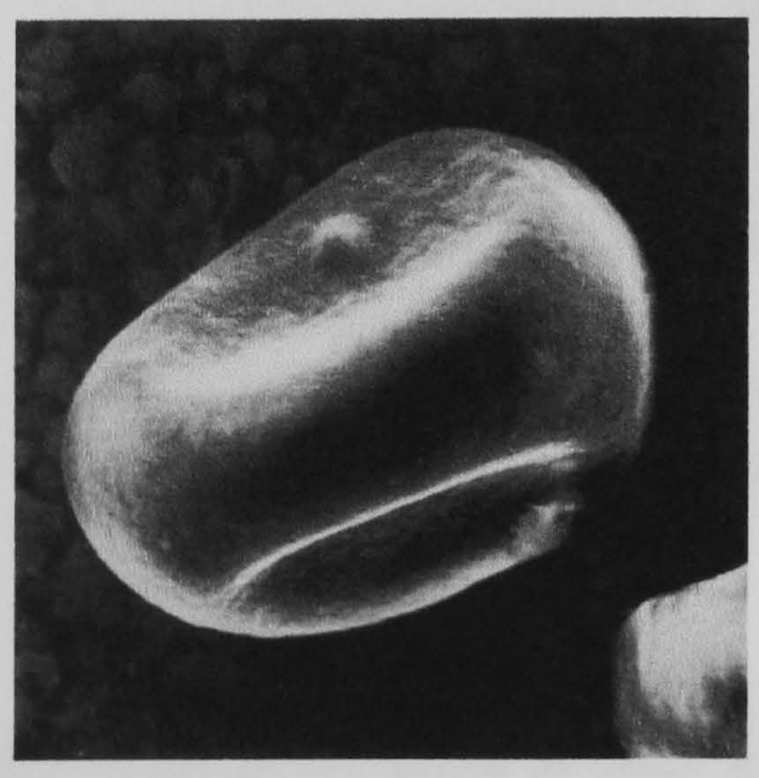
Biodegradability of Sporopollenin

Jorde and Linskens (1974)[53] found that after human subjects were fed 100-150 g of either spores from *Lycopodium clavatum* or pollen from *Secale cereale*, both types of particles were recovered in the blood. With the aid of light and scanning electron microscopy, the maximum amount of pollen/spores found in venous blood was 30 minutes after ingestion. It was also observed that the pollen and spores underwent degradation in the blood. From the microscope studies it was shown that after 30 minutes the exine became covered with a film of serum and the shapes became smoother. After 90 minutes, the exine started to decay and after 120 minutes only the debris of the exine was visible. Figure 3.10 shows pollen before ingestion and pollen taken from the

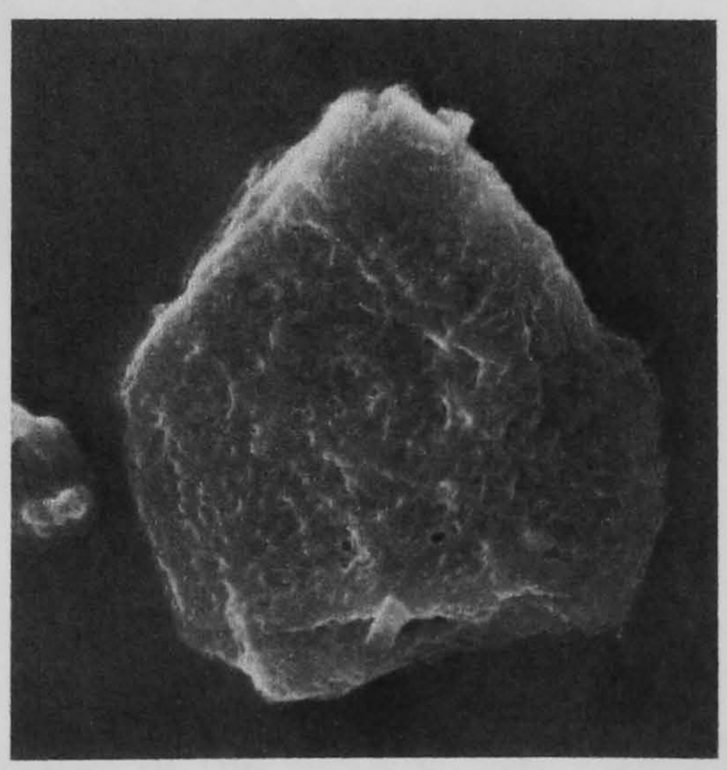
blood 90 minutes after ingestion. This degradation process seemed proportional to the length of time the particles remained in the blood. It is believed that enzymes in the blood are capable of breaking down pollen and spores. In contrast, and suprisingly, *in vitro* incubation of spores in blood showed no decay.

Studies have also shown that pollen fed to dogs was also found in the blood and in the urine and cerebral spinal fluid of most dogs.[5]

The passage of large particles such as pollen and spores in this way is termed persorption.[53-56] Persorption can be defined as "the passage of large, solid particles, well within the μm range, through the epithelial cell layer of the intestinal wall." Other particles such as starch granules, cellulose particles and fibres are all capable of paracellular uptake from the digestive tract and are transported away by the lymph and blood vessels.[57] These persorbed particles have also been detected in urine, bile, cerebrospinal fluid, breast milk and are transported across the placenta into foetal blood.[57]



(A)



(B)

Figure 3.10 Scanning electron micrographs of (A) Rye pollen before ingestion and (B) and Rye pollen recovered in the blood 45 minutes after oral ingestion.[5,53]

Human and animal studies have shown that large particles are "kneaded" through the epithelial layer passing between adjacent cells.[55-57] Volkheimer *et al* (1967)[58] and Fabian (1983)[56] demonstrated visually the phenomenon of persorption using light microscopy. Experiments on various animal species using particles of μm size, showed that after ingestion these particles moved away from the surface of the villi of the intestinal wall and could be found in the blood and urine after 10 minutes.

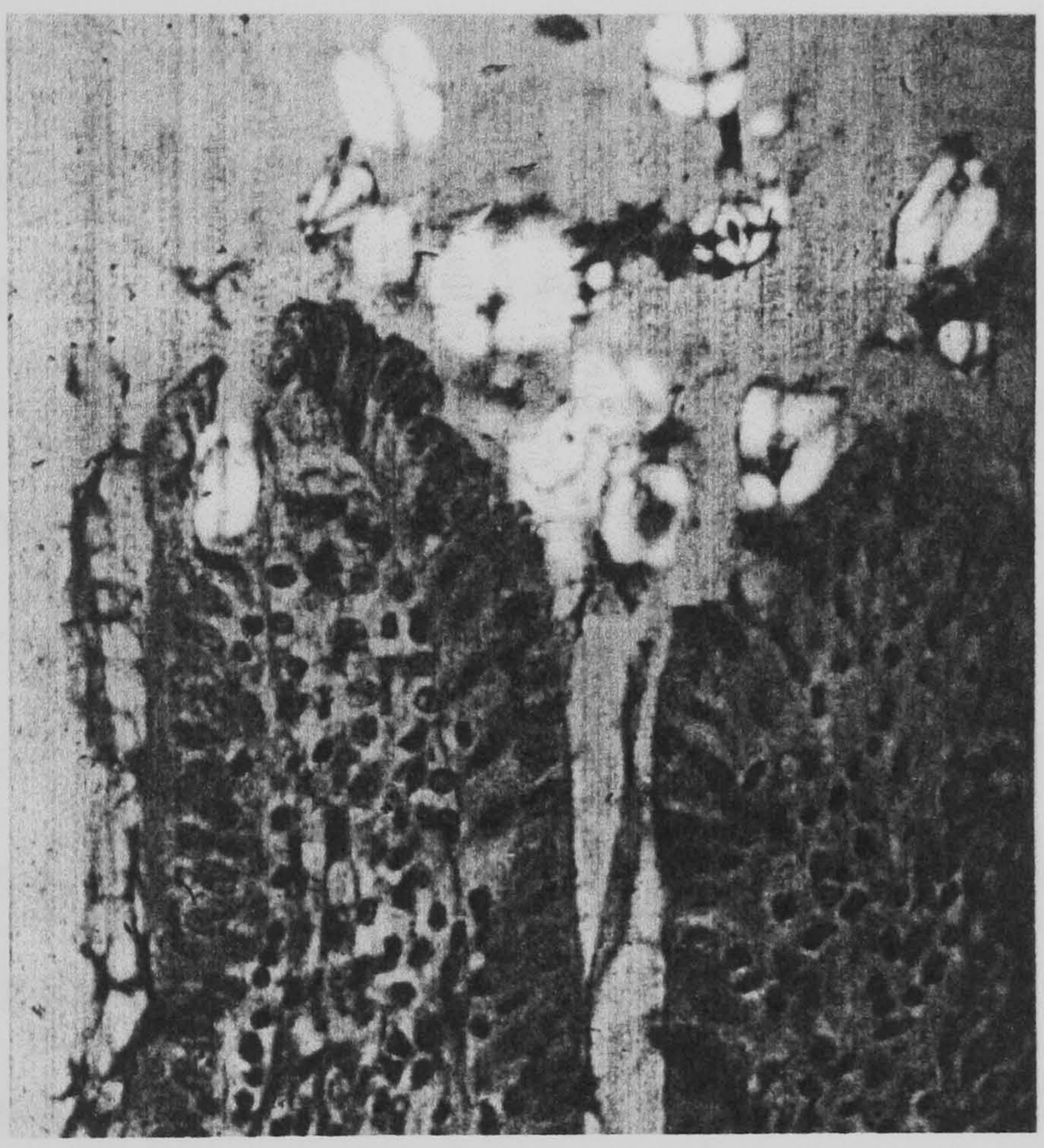


Figure 3.11 The persorption of large granules in the small intestine of the rat.[58]

Figure 3.11 shows the persorption of large granules at the apex of the villus between the enterocytes in the subepithelial region in the jejunum of the rat.[58]

When Volkheimer *et al* (1968)[55] carried out studies on human subjects using potato starch (particle size 8-110 μm) they found that only a few starch granules remained in the

blood 8 hours after ingestion and after 3 days it was practically impossible to find starch granules circulating in the blood.[55]

These series of experiments have demonstrated that pollen and spores are capable of undergoing biodegradation in the blood. Degradation and collapse of these materials seems to occur between 90 and 120 minutes. Although sporopollenin or functionalised sporopollenin have not been studied in this way, it seems likely that they would behave in the same way as the whole pollen/spore when ingested orally, as microscopic evidence has shown that the exine (sporopollenin) becomes degraded into small fragments.

Non-Toxicity of Sporopollenin

Pollen and spores mixed with honey or in tablet form are available as a natural food source and has been used by athletes as a highly concentrated food source.[5] Pollen has been found to relieve chronic prostasis (inflammation of the prostate)[5] and has been prescribed for the irritability of the bladder, which is reported to be official in the United States pharmacopea.[59] It has also been reported that allergic reactions, such as hayfever, may be reduced by oral ingestion of pollen and pollen tablets are available from health shops for the treatment of hayfever.

The spores of *L. clavatum* have been used since the 17th century for internal complaints such as diarrhoea and dysentry.[60] It has also been used by homeopathic physicians in dilute doses for the treatment of digestive disorders.[60,61] *Lycopodium clavatum* spores are still used today and can be purchased from many health shops. *Lycopdium* spores have also been used as a dusting powder for pills[59,61] and as a bulking agent in sweets.[53]

There has been no information found in the literature to suggest that ingestion of large quantities of pollen or spores has any harmful or toxic effects on humans and animals. The information found in the literature only points to the observation that pollen and

spores are non-toxic and can be taken orally with no ill effects. Additionally, persorption of other particles such as starch and cellulose are consumed daily in our food with no adverse effects and the persorption process is considered to be a secondary feature of the digestive process.[55]

As pollen and spores are considered non-toxic it would seem reasonable to say the sporopollenin also would be non-toxic. The functionalised sporopollenin used for drug delivery would also have to possess functional groups which upon degradation would be not be toxic.

Stability of Functionalised Sporopollenin

Sporopollenin needs to be appropriately functionalised to permit efficient attachment of the drugs. The drug-sporopollenin attachments need to be stable to the acidic conditions present in the stomach in order for it to pass into the small intestine intact where it can be absorbed into the bloodstream.

3.5.5 Aims and Objectives for the Application of Sporopollenin in Drug Delivery

In order to determine the potential usefulness of sporopollenin in drug delivery, the following investigations are proposed:

a) Sporopollenin will need to be derivatised with a range of non-toxic linkers to find the most appropriate functionalisation for the efficient attachment of drug molecules.

b) The loadings of these functional groups will need to be determined and the loading of the drug attached to sporopollenin needs to be reasonably high in order to minimise the amount of sporopollenin required to obtain a therapeutic level. This could be achieved by attachment of non-toxic polyfunctional linkers.

c) The functionalised forms of sporopollenin will need to be assessed for their stability to normal gastric conditions.

d) Attempts to attach drugs molecules to sporopollenin *via* functional groups will be undertaken and the loadings of the drugs assessed.

e) The stability of these drug attachments to gastric conditions will need to be investigated.

f) *In vivo* experiments will then have to be carried out to see if sporopollenin with the drug attached after ingestion will pass intact from the stomach to the small intestine where it then can be absorbed into the blood stream where the drug will hopefully be then released.

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4 RESULTS AND DISCUSSION

4.1 PREPARATION OF SPOROPOLLENIN

A number of methods to prepare sporopollenin from *Lycopodium clavatum* were attempted ranging from harsh treatment with acids to more gentle treatments using enzymes (See Chapter 5, Section 5.3). These methods were compared for their efficiency and are discussed below.

METHOD A

This method of preparing sporopollenin included the following essential steps:[1-3]

a) The treatment of the spores with hot organic solvents which removed surface coatings and a small amount of fat.

b) A KOH reflux which served to release the cytoplasmic contents such as proteins, fats and waxes.

c) Solvent and aqueous washes which removed the breakdown products.

d) Treatment with 85% H_3PO_4 which removed the intine (cellulose layer).

After the various treatments and washings a brown powder remained which was considered to be the chemically resistant exine of sporopollenin.

An IR spectrum of the final sporopollenin prepared using Method A (Appendix 1, IR 1) revealed a complex spectrum with more peaks present than in the IR spectrum of *L.clavatum* sporopollenin prepared by G. Shaw (Chapter 3, Section 3.3.3.1, Figure 3.5, Spectrum C). It was possible that these extra peaks could have been due to trapped reagents and solvents such as phenol which had not been completely removed during the washing procedures. IR absorption frequencies corresponding to aromatic C=C

stretching around 1595 to 1400 cm^{-1} and C-H bending deformations around 750 to 692 cm^{-1} indicated the presence of phenolic impurities.

To see if this suspicion was true, a sample of sporopollenin was re-washed with ethanol, water, ethanol and DCM and re-dried. The IR spectrum (Appendix 1, IR 2) of the re-washed sporopollenin showed that these peaks mentioned above had disappeared. This indicated that it was difficult to wash out reagents such as phenol from the sporopollenin even when large volumes of solvents were used. Elemental analysis of the sporopollenin prepared by Method A revealed that there was a small amount of nitrogen present after processing (See Figure 4.1). As the procedure involved a step with ninhydrin to remove any residual amino acids it was assumed that any nitrogen remaining in the sporopollenin was inaccessible or unreactive. See Table 4.1 for elemental analysis.

METHOD B

Because it was found to be difficult to completely wash away phenol from the sporopollenin it was decided to miss out the steps in the procedure which involved phenol. The step involving ninhydrin was also missed out as this also involved the use of phenol. The IR spectrum (Appendix 1, IR 3) showed no significant differences to the sporopollenin prepared by Method A. Elemental analysis can be seen in Table 4.1.

METHOD C

There have been various methods of preparation of sporopollenin in the literature (See Chapter 3, Section 3.2) and most researchers adopt their own method of preparation. It was decided to miss out some of the steps used in processing of sporopollenin Method A. This was carried out for a number of reasons:

a) Using fewer steps would decrease the length of time required to obtain the sporopollenin.

b) The use of fewer reagents would keep the cost of preparation to a minimum.

c) The less reagents used, the less likely it was to introduce impurities or chemically modify the sporopollenin structure.

This method of preparation was carried out a number of times. Results of the elemental analyses are shown in Table 4.1.

Method	**Sporopollenin Sample**	**% Elemental Analysis**					**C_{90} Formula**	**IR Spectrum**
		C	H	O	N	S		
A	01[a]	62.28	7.27	30.28	0.17	0	$C_{90}H_{125}O_{32}$	1,2
B	02[a]	68.22	8.24	23.34	0.21	0	$C_{90}H_{130}O_{23}$	3
C	03[b]	74.21	7.94	17.72	0.13	0	$C_{90}H_{115}O_{16}$	4
C	04[c]	63.31	6.67	29.81	0.21	0	$C_{90}H_{113}O_{32}$	5
C	05[c]	66.22	8.16	25.58	0.15	0	$C_{90}H_{132}O_{26}$	6
C	06[b]	75.47	7.58	16.90	0.05	0	$C_{90}H_{108}O_{15}$	7
C	07[c]	64.28	6.27	29.37	0.08	0	$C_{90}H_{104}O_{31}$	8
C	08[d]	64.95	5.47	29.88	0.06	0	$C_{90}H_{91}O_{32}$	9
C	09[d]	66.27	5.69	27.95	0.09	0	$C_{90}H_{92}O_{29}$	10
D_1	10.1[d]	62.70	5.16	27.39	0	4.75	$C_{90}H_{88}O_{30}S_3$	11
D_2	10.2[d]	56.04	4.80	36.70	0	2.46	$C_{90}H_{92}O_{50}S_2$	--
D_3	10.3[d]	51.14	4.37	43.02	0	1.48	$C_{90}H_{92}O_{57}S_1$	--
E	11[d]	56.30	8.38	33.64	1.67	1.67	$C_{90}H_{159}O_{41}N_3$	12

Table 4.1 Elemental analysis results for the sporopollenin samples.
Subscript letters refer to the different batches of starting Lycopodium powder used.
C_{90} formulae were used to compare with literature values.
The nitrogen was omitted form the C_{90} formulae from samples 01-09 as the amount was less than 0.2.
Samples 04, 05 and 07 were prepared by exchange students.[27a-c]

The literature values of C, H and N analysis of sporopollenin are $C_{90}H_{144}O_{27}$[4] and $C_{90}H_{136}O_{28}$.[5]

For consistency, it was essential that the method of preparation was strictly followed to minimise any differences in the sporopollenin due to deviations from the original method. The samples of sporopollenin, 03 to 09, varied slightly in their elemental composition which seemed dependent upon the batch of Lycopodium powder used. Samples of sporopollenin prepared using different batches of Lycopodium powder also had slight differences in their IR spectra (See Appendix 1, IR 4-10) The IR region between 1700 and 1000 cm^{-1} differed in the number and intensity of absorptions. Table 4.2 shows the variation in elemental composition of the sporopollenin samples depending on which batch of Lycopodium powder was used.

Sporopollenin Sample*	C_{90} Formula	Lycopodium Batch	IR spectrum
03	$C_{90}H_{115}O_{16}$	b	4
06	$C_{90}H_{108}O_{15}$	b	7
04	$C_{90}H_{113}O_{32}$	c	5
05	$C_{90}H_{132}O_{26}$	c	6
07	$C_{90}H_{104}O_{31}$	c	8
08	$C_{90}H_{91}O_{32}$	d	9
09	$C_{90}H_{92}O_{29}$	d	10

Table 4.2 Comparison of sporopollenin (Method C) prepared using different batches of Lycopodium powder.
* See Table 4.1.

METHOD D

It was found that the number of hydroxyls present in sporopollenin differed between the samples prepared using the same method (See Section 4.4.1, Chapter 4). This variation

in hydroxyl content seemed to be linked to the different batches of Lycopodium powder purchased. Shaw and Apperley[6] (1996) demonstrated that the use of orthophosphoric acid commonly used to remove cellulose was unsatisfactory. The ^{13}C-NMR spectra of sporopollenin derived from *Lycopodium clavatum* spores prepared by two separate seven day treatments with 80% H_3PO_4 at 80-90°C indicated that the cellulose was still present. After an extra treatment with 80% H_2SO_4 the cellulose seemed to be completely removed. The differences in hydroxyl content of the sporopollenin samples could have been due to incomplete removal of cellulose during the treatment. It was therefore decided to incorporate an extra treatment with 80% H_2SO_4 in the procedure. A portion of the sporopollenin sample 06 was subjected to treatment with 80% H_2SO_4 at 80° C for 5 days. After this treatment the sporopollenin turned a charcoal colour. The IR spectrum (Appendix 1, IR 11) again differed slightly in the absorptions between 1700 and 1000 cm^{-1}. Elemental analysis (Sample 10.1) showed that all nitrogen had been removed but sulfur had been incorporated into the sporopollenin. This was in agreement with the findings by Zetzsche *et al* who found that sulfur was introduced into sporopollenin after treatment with 72% H_2SO_4.[1] The sulfur present in sporopollenin after treatment with the acid could have arose from the formation of esters of sulfuric acid upon reaction of the hydroxyls present in sporopollenin. Normally sulfuric acid esters can be readily saponified by refluxing with water or dilute alkali. Hence, a sample of the sporopollenin prepared using Method D1 was refluxed with 2M NaOH and indeed it was found by Elemental analysis (Sample 10.2) that the sulfur content had been reduced. Treatment of the sporopollenin with refluxing KOH reduced the sulfur content even further (Sample 10.3). After these treatments any remaining sulfur was considered to be unreactive.

Large scale preparation of sporopollenin incorporating a sulfuric acid treatment was carried out. Problems were encountered during filtration and washing after the step with sulfuric acid. Filtration was very slow through a grade 3 porosity sintered funnel leading to eventual clogging of the sinter. At this stage it was suspected that the spores had been degraded and broken down into smaller particles during this vigorous acid treatment. The sporopollenin when viewed under a light microscope showed signs of degradation. The sporopollenin was either broken up, distorted or reduced in size. It was therefore concluded that this method of preparation was unsuitable and no further treatments with sulfuric acid were carried out.

METHOD E

This procedure used 4-MMNO.H_2O and cyclohexylamine to swell the spores and loosen the exine layer.[7] The protoplasts were released and treatment with cellulase and pectinase enzymes destroyed the attachments between the intine and exine layers. The sporopollenin was then separated from the other parts of the spore. Sporopollenin isolated using this method remained bright yellow in colour. The advantages of this method was that it can be operated at room temperature with the use of mild reagents and it is quick and simple.[7] The IR spectrum (Appendix 1, IR 12) differed once again in the absorptions between 1700 and 1000cm^{-1}. Elemental analysis showed that there was a larger portion of nitrogen remaining in the sporopollenin than that with sporopollenin prepared using Methods A to D. This could have been due to the incomplete removal of nitrogen containing spore material, such as amino acids, the failure to wash all 4-MMNO and cyclohexylamine from the sporopollenin or the possibility of these reagents reacting with the sporopollenin. Other drawbacks of this method were that only small amounts of

L. clavatum spores could be processed at any one time and the reagents were expensive. Both these disadvantages made this method not suitable for preparation on a larger scale. The other method of isolation of sporopollenin reported in the literature involving the use of anhydrous HF[8] was not attempted because specialist equipment would be required and because HF is very toxic and corrosive it would only be acceptable on small scale isolations.

The conclusions were that Method C was the preferred choice because it produced sporopollenin which remained intact and free of entrapped impurities. Also, the procedure was shorter than Methods A, B and D and it could be conveniently scaled up unlike Method E. The reagents used in the preparation were also cheap and readily available.

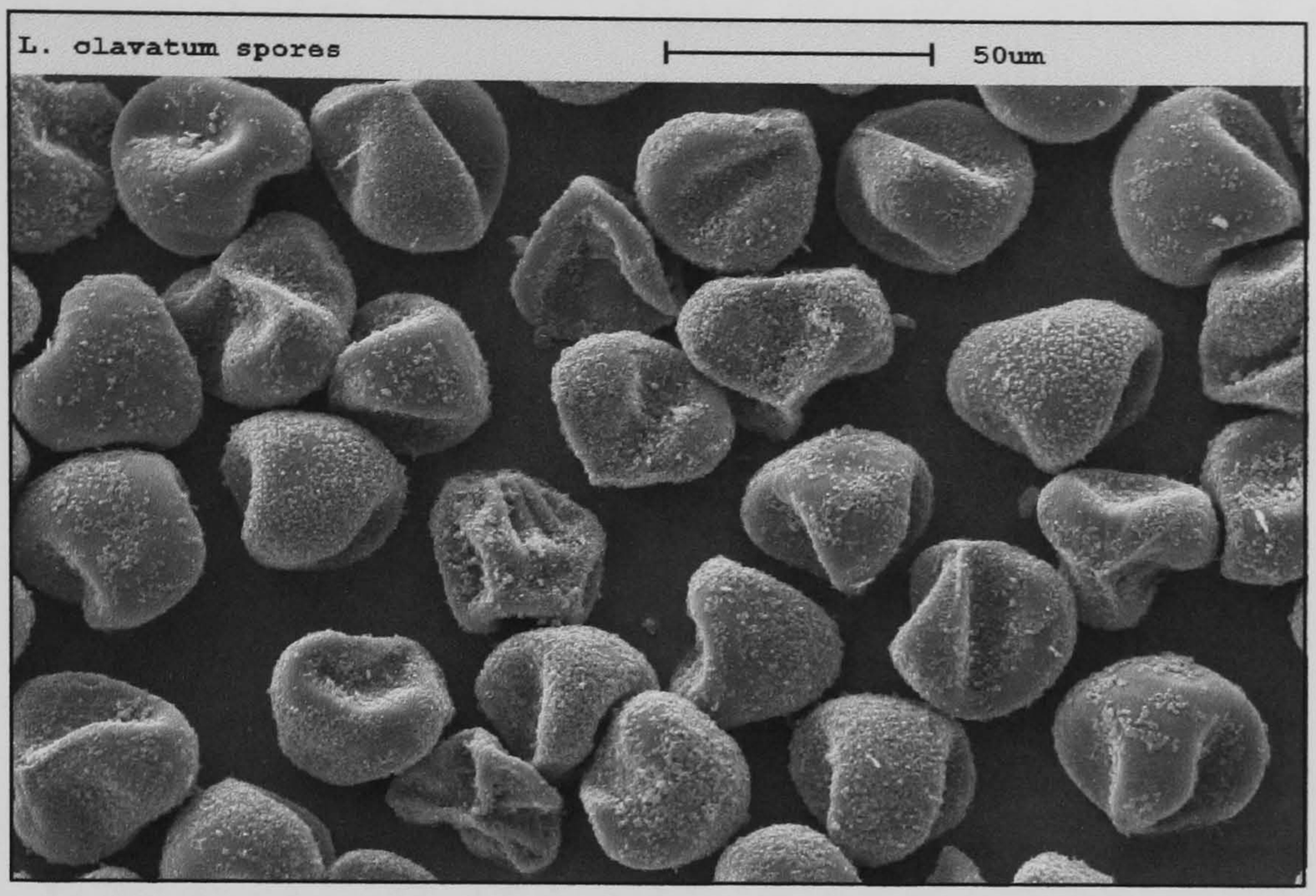
4.2 PHYSICAL STUDY OF SPOROPOLLENIN

A number of experiments were carried out to examine the swelling, filtering and drying characteristics of sporopollenin. Sporopollenin is found to have favourable properties which could make it a suitable material for applications in the areas of solid phase synthesis and drug delivery. These properties have been discussed in Chapter 3, Section 3.5.

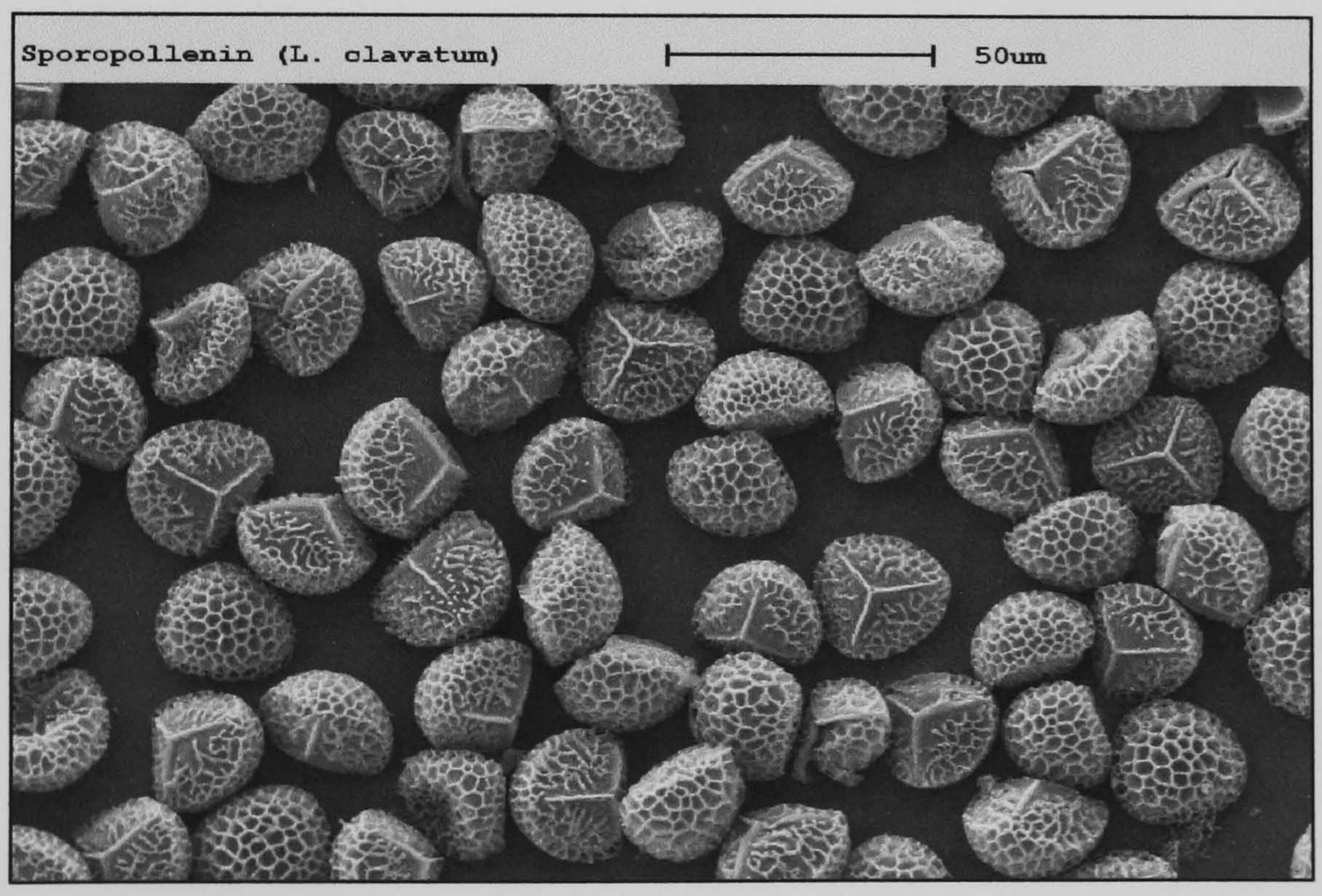
4.2.1 Physical Appearance

SEM images of *Lycopodium clavatum* spores and sporopollenin prepared using Method C were obtained. Figure 4.1 show SEM images of *Lycopodium clavatum* spores and sporopollenin after treatment. The spores became flattened and compressed under vacuum. The spores had tiny granules scattered on the surface and small fragments and

debris were visible around the spores. Most of the sporopollenin remained intact. Some had burst slightly or had become distorted but this was probably due to the high vacuum encountered in the electron microscope. On one side of the spore exine there is a three-branched slit forming a Y shape clearly visible. This is called the trilete scar.[3] The spores are defined as rounded/triangular and the surface is highly sculptured with a network of raised ridges on the surface with gaps which look honeycomb in structure. No fragments or debris were visible in the samples.



(A)



(B)

Figure 4.1 Scanning Electron Micrographs of:
(A) *Lycopodium clavatum* spores before treatment
(B) Sporopollenin produced by Method C.

Light microscope images can be seen in Figures 4.2 to 4.4.

Sporopollenin prepared using Method C (Figure 4.2) remained intact and retained the full morphology of the original spore. No breakdown of the sporopollenin structure occurred.

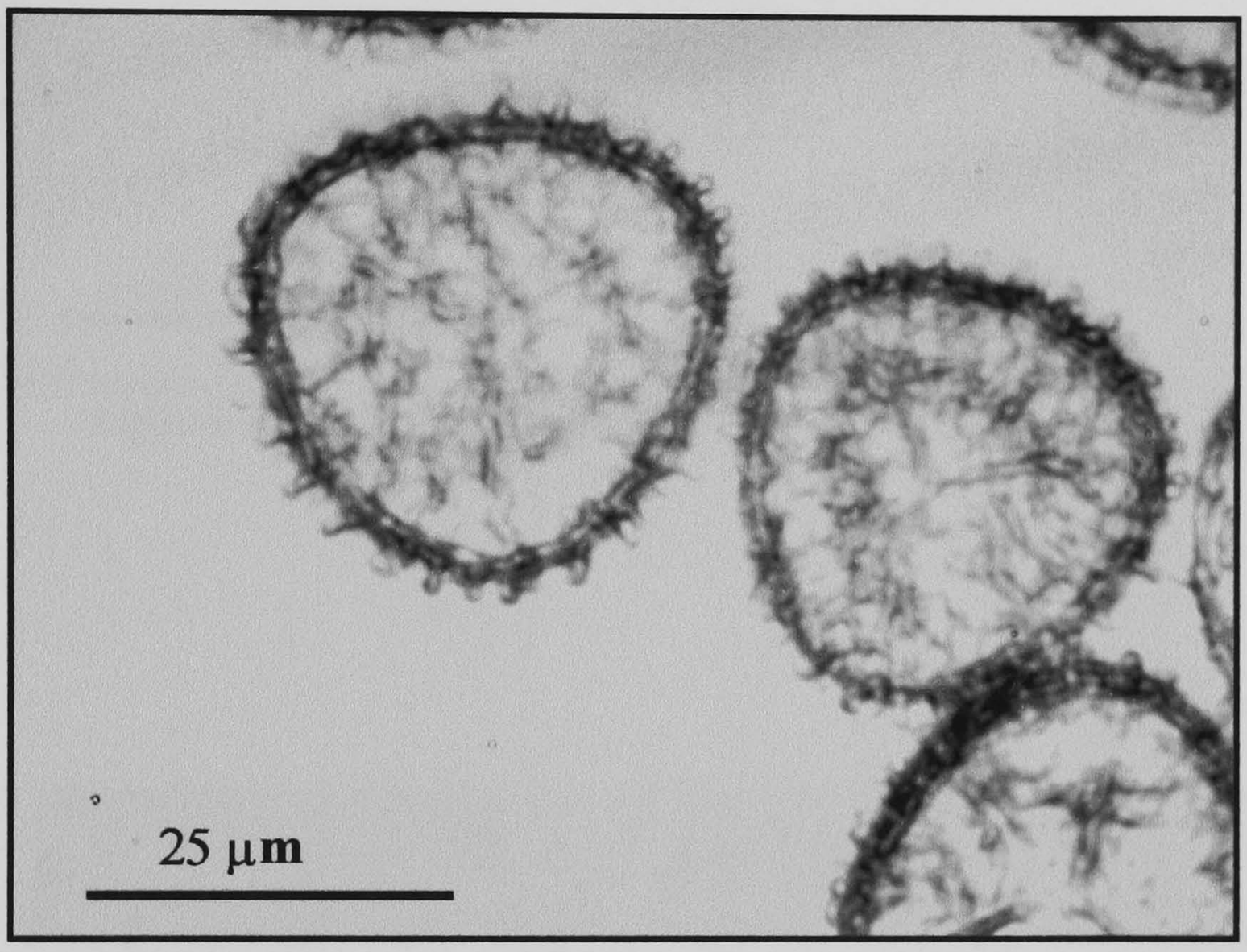


Figure 4.2 Sporopollenin prepared using Method C.

Sporopollenin prepared using Method D had clearly degraded during the sulfuric acid treatment. The sporopollenin had shrunk, become darker and fragmented. This can be seen in Figures 4.3 and 4.4.

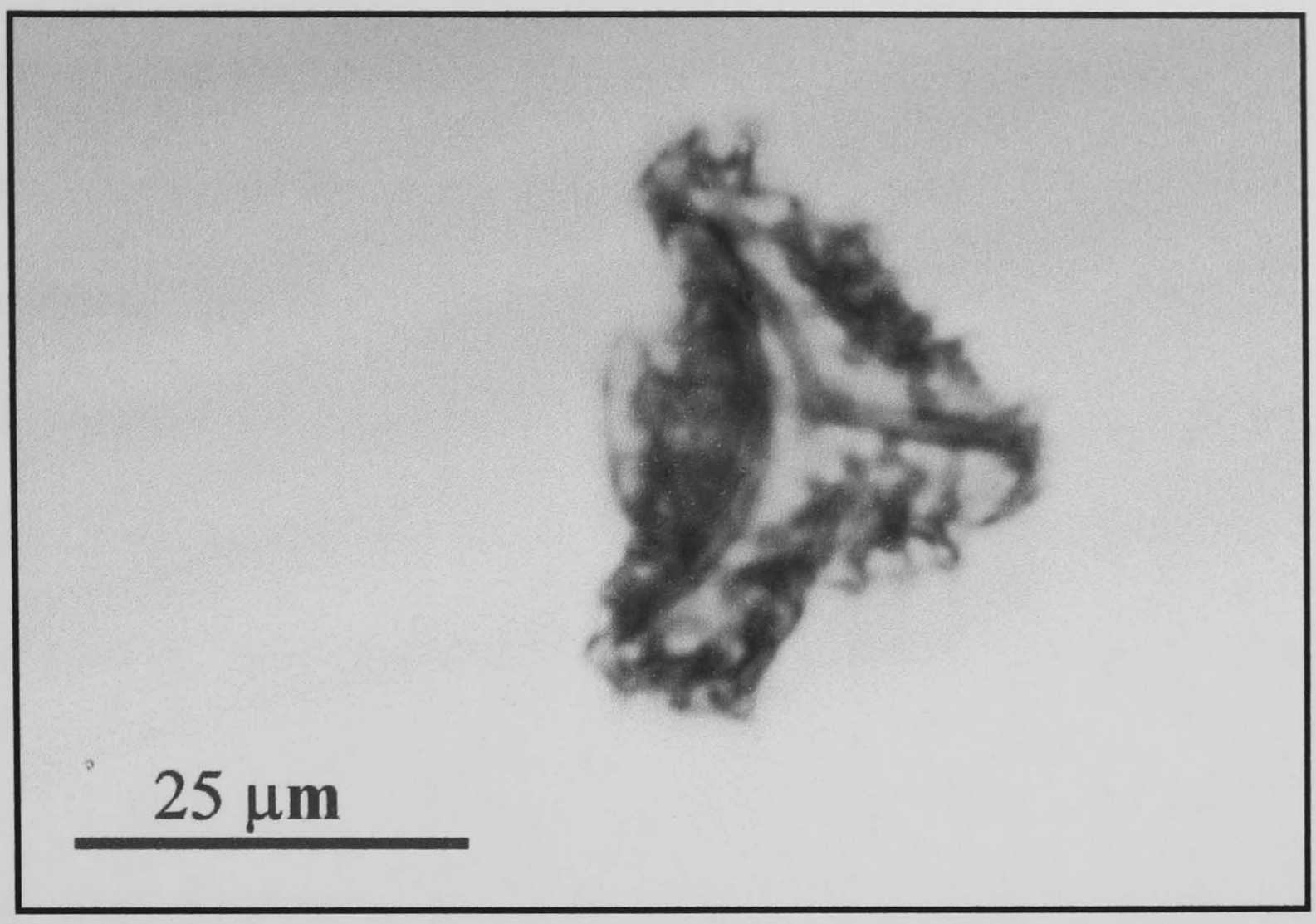


Figure 4.3 Sporopollenin prepared using Method D.

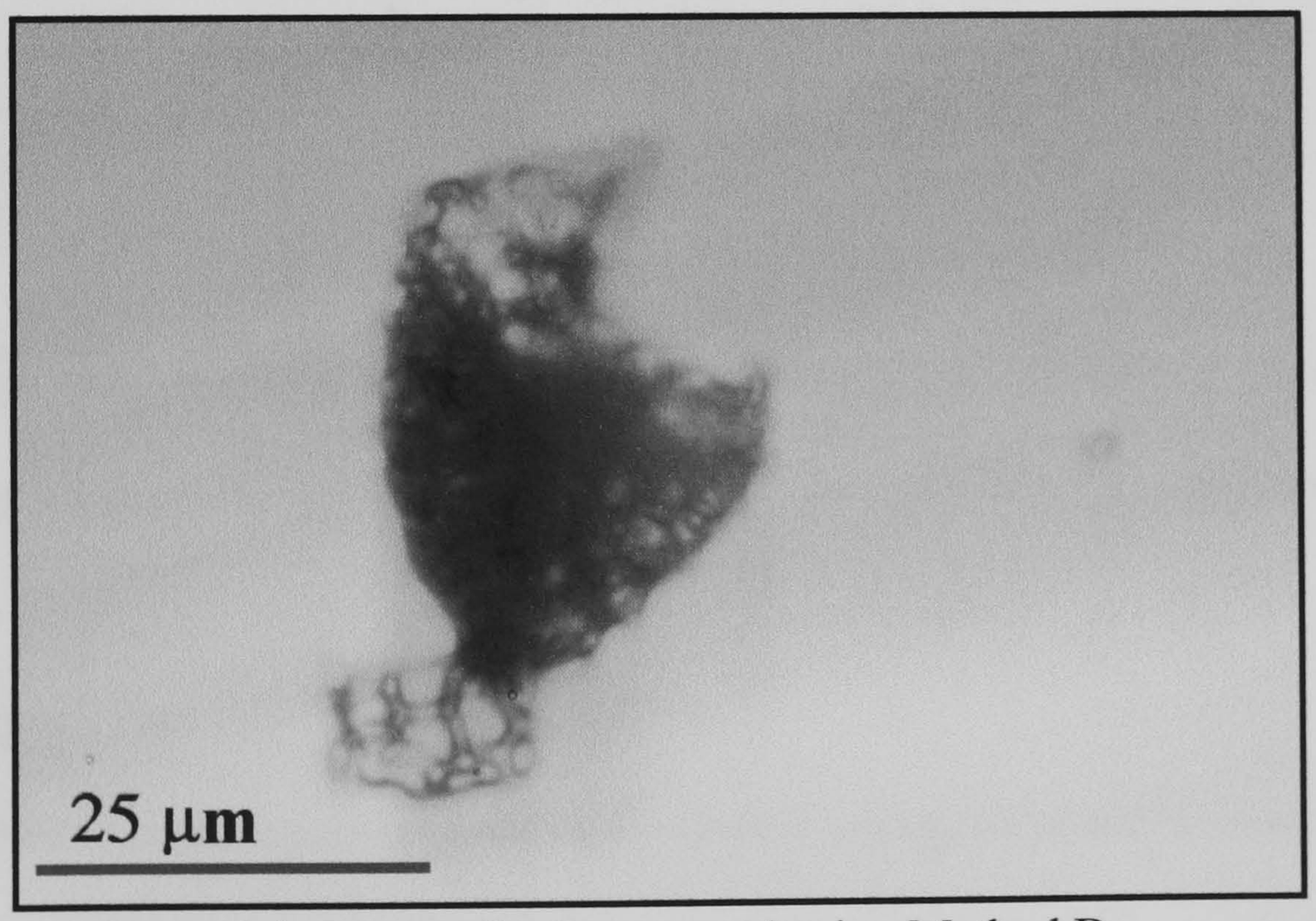


Figure 4.4 Sporopollenin prepared using Method D.

4.2.2 Swelling characteristics

Lycopodium spores and sporopollenin were placed in various solvents to check for any swelling. The diameters of the spores and sporopollenin were measured in μm. Table 4.3 shows the average diameters of the *Lycopodium* spores and sporopollenin in various solvents. The literature value of sporopollenin has been reported to be around 20 μm.[9] From the results it can be seen that the sporopollenin swells slightly in the solvents tested. Of the solvents investigated, methanol and DCM make the sporopollenin swell the most.

Solvent	Diameter of *Lycopodium clavatum* spores (μm)	Diameter of sporopollenin (μm)
Water	42	25
DMF	40	28
DCM	42	32
Methanol	42	32

Table 4.3 Average diameters of *Lycopodium clavatum* spores and sporopollenin. The values are the mean of 10 individual measurements.

Because sporopollenin does not swell in aqueous and organic solvents it has a major advantage over microporous-gel type supports which have to be swollen prior to use.

4.2.3 Filtration

It was established that sporopollenin was easily filtered with grade 3 and 4 sinters from each of the solvents DMF, DCM, THF, ether, methanol and water with no resistance or clogging of the sinter. The sporopollenin did not break down and hinder filtration by the generation of fines. Table 4.4 shows the increased filtration times of the solvents when

sporpollenin was present compared to the time taken for the solvents alone to pass through the sinters.

The sporopollenin was too fine for a grade 1 sinter as it passed through the large pores present in the sinter. When using a grade 2 sinter, filtration was longer due to clogging of the sinter with the sporopollenin particles.

Porosity of sinter (BS Grade)	Pore Size of Sinter (μm)	Increased Filtration Time in Presence of Sporopollenin
1	100-160	Sporopollenin passed through
2	40-100	1 - 7
3	16-40	1 - 3
4	10-16	1 - 3

Table 4.4 Filtration times for various solvents in the presence of sporopollenin.

The filtration times were also dependent on the solvent used. Table 4.5 shows the increased filtration rates of the solvents due to the presence of sporopollenin. From these results it was established that grade 3 or 4 sinters were the most efficient when collecting sporopollenin by filtration and that the solvents DCM and diethyl ether gave the fastest filtration rates. Similar results were found with Alltech® tubes which are often used in solid phase organic synthesis.

Solvent	Increased filtration time for the solvent in the presence of sporopollenin
DCM	1 - 2
Diethyl ether	1 - 2
Water	1 - 3
Methanol	2 - 3
THF	2 - 3
DMF	3 - 7

Table 4.5 Filtration rates of various solvents.

4.2.4 Drying Experiments

After an initial drying in the air and then drying overnight in a vacuum dessicator over P_2O_5 overnight, sporopollenin samples were dried using a number of methods.

1 With the use of a **vacuum oven** at 80°C in the presence of P_2O_5, sporopollenin was dried to a constant weight for 72 hours (3 days) to give a weight loss of 5%.

2 When sporopollenin was dried in a **microwave oven** for 1 hour the weight loss was 4.8%.

3 With the use of a **vacuum dessicator** and P_2O_5 the sporopollenin reached a constant weight after 120 hours (5 days). The weight loss was found to be 4.9%.

Table 4.6 compares the three drying methods used. All the drying methods gave around 5% loss on drying.

Drying Method	Time Taken to Dry to a Constant Weight (Hours)	Weight Loss (%)
Vacuum Oven, 80°C, P_2O_5	72	5.0
Microwave, 90°C	1	4.8
Vacuum Dessicator, P_2O_5	120	4.9

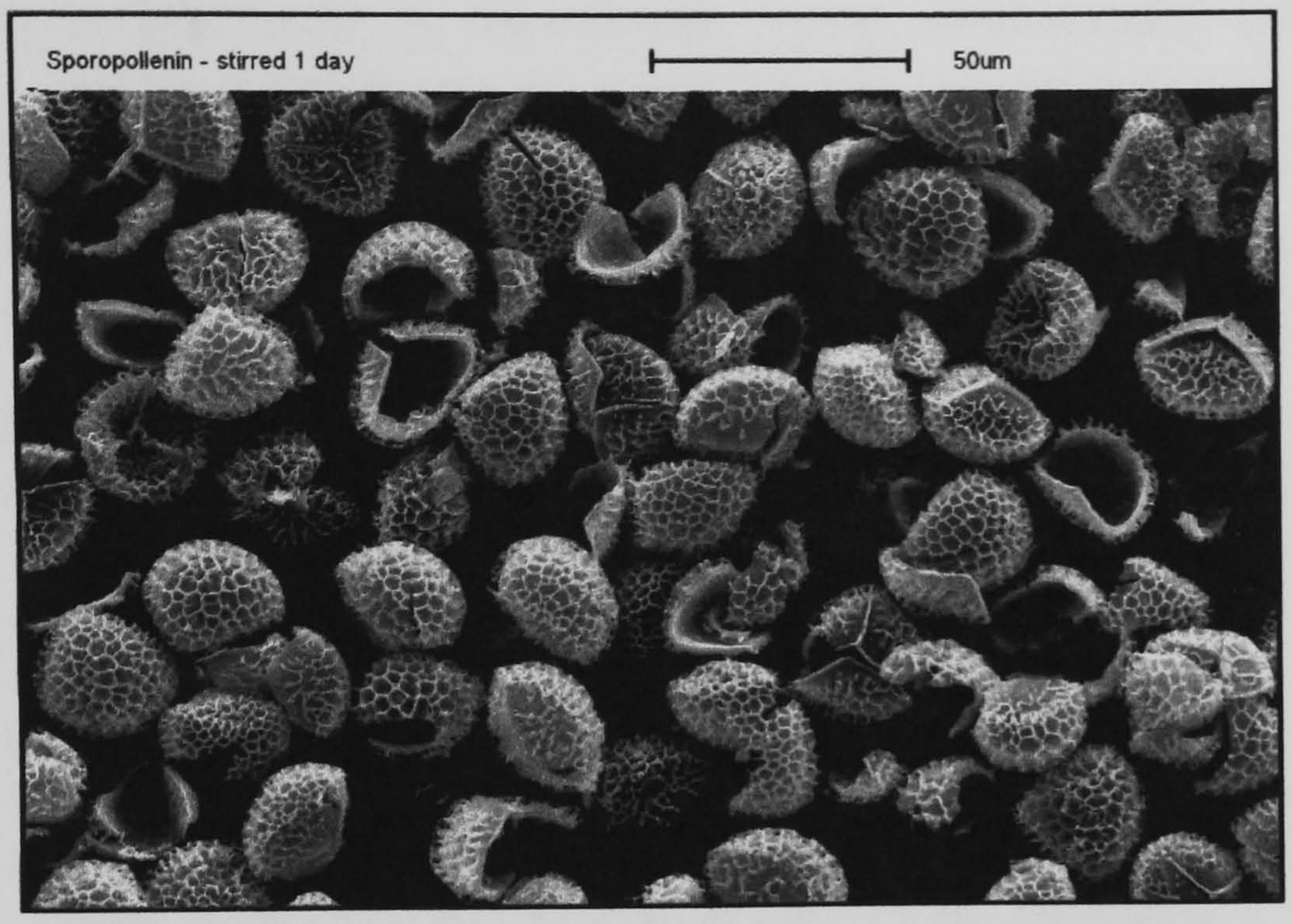
Table 4.6 Comparison of the drying methods for sporopollenin.

The method of choice for drying sporopollenin was the use of a vacuum dessicator at room temperature or use of the vacuum oven. Drying in the microwave oven was the most efficient but was not used routinely because of the possibility of damage to the sporopollenin structure by the microwaves. Arens and Traverse (1989)[10] suggested that since microwaving was known to cause steam-induced explosion of sealed, moist systems such as eggs, potatoes and popcorn kernels, it was possible that pollen grains and spores containing water would behave in the same manner. Microscopic examination of fresh spores and pollen and those processed by treatment with KOH or acetolysis by microwaving does rupture these structures.[10]

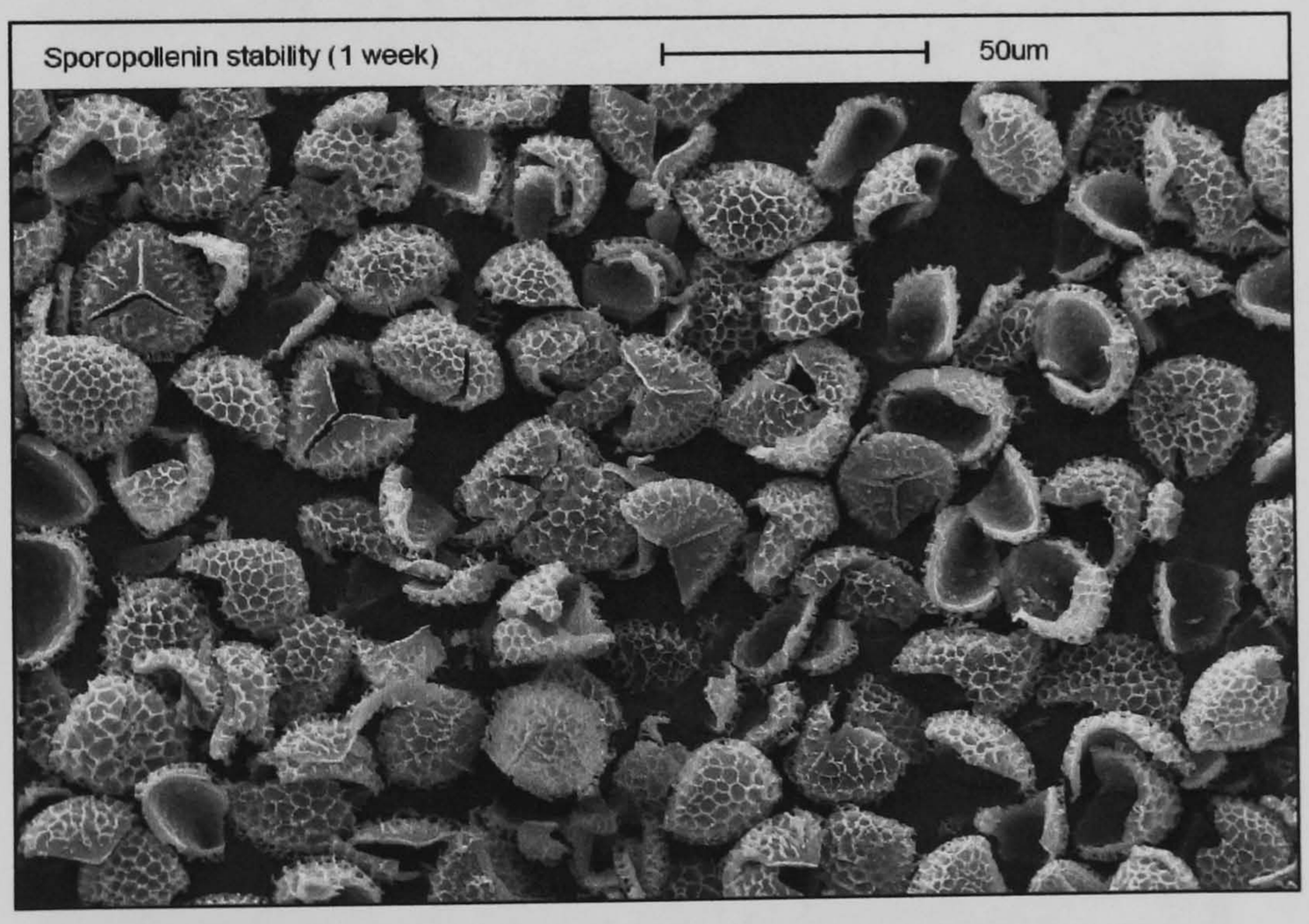
4.2.5 Mechanical Stability

SEM images of sporopollenin, prepared by Method C, stirred magnetically for 1 to 7 days were taken. Figure 4.5 shows SEM images of sporopollenin after magnetic stirring after 1 day and 1 week. The SEM images revealed that the sporopollenin had started to degrade after one day. This degradation seemed to level off after 4 days. Some sporopollenin had been degraded into smaller fragments but it was still easily filterable and did not appear to block the sinter during filtration even after stirring for 1 week. In contrast, commercial Wang resin[11a] (100-200 mesh) was magnetically stirred for 1 day

and was found to be severely degraded. This fragmented Wang resin was difficult to filter and blockage of the sinter occurred.



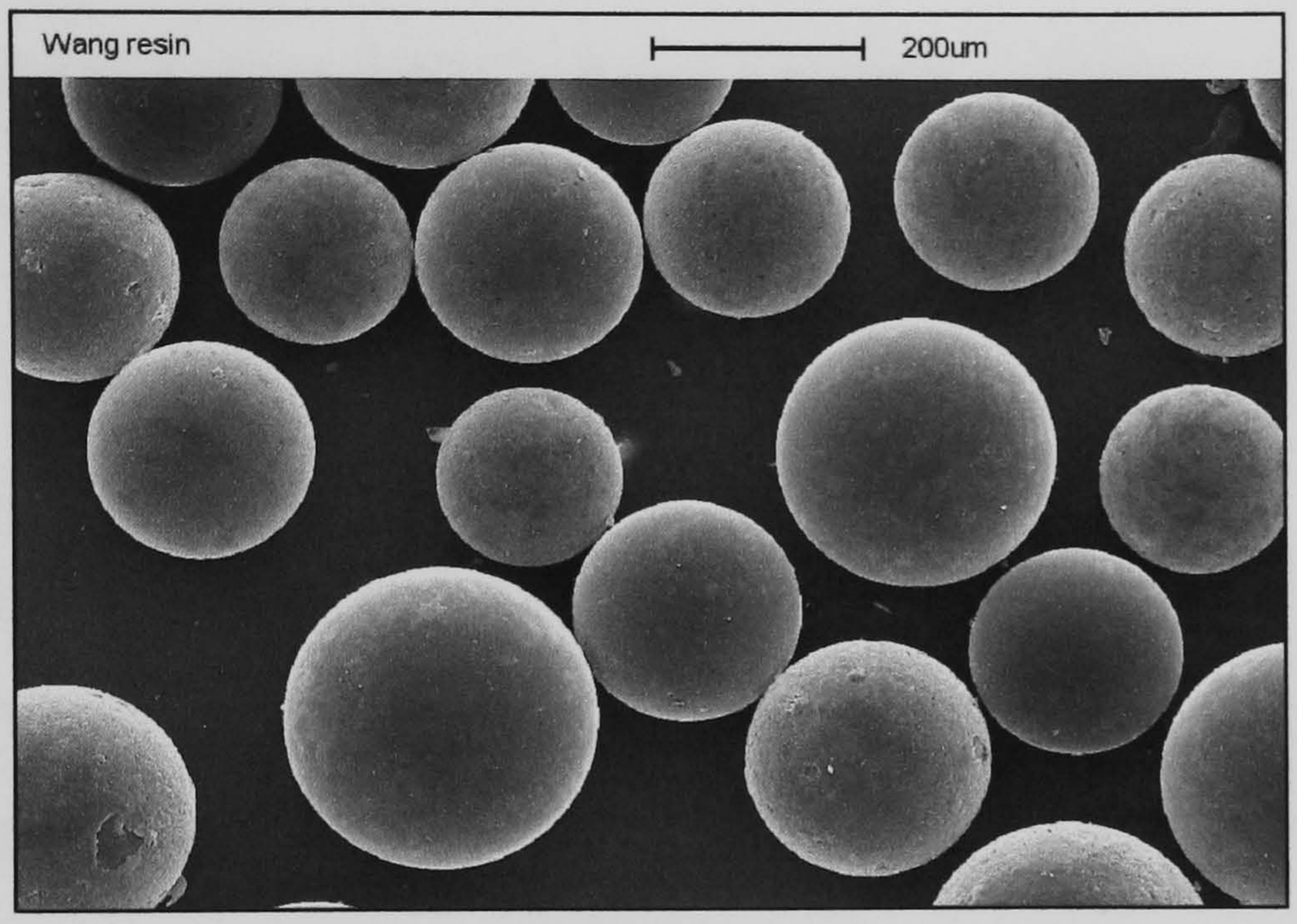
(A)



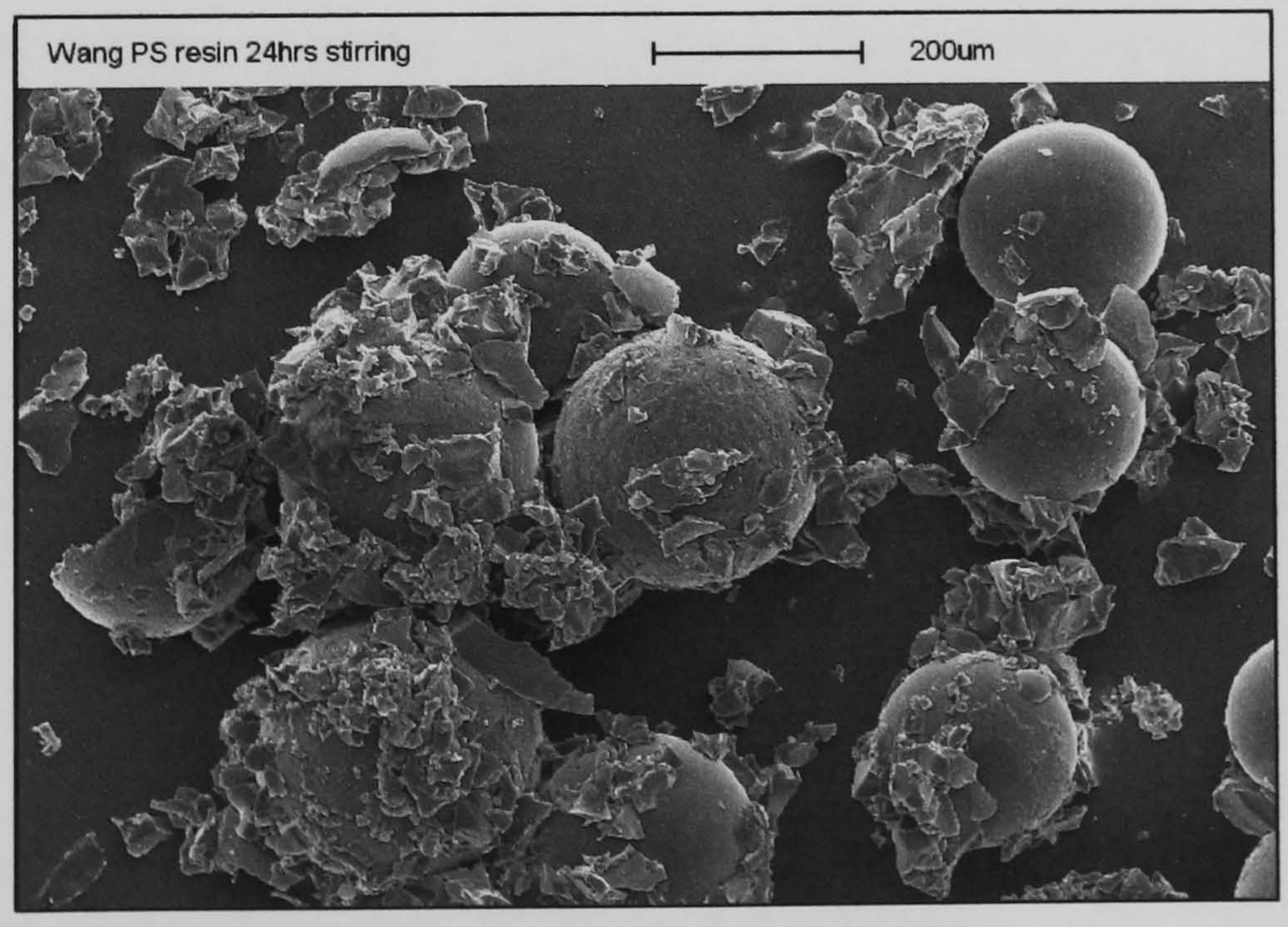
(B)

Figure 4.5 SEM images of Sporopollenin (Method C)
(A) After stirring for 1 day (24 hours)
(B) After stirring for 1 week.

Figure 4.6 shows SEM images of Wang resin before stirring and after stirring for 1 day. Some of the Wang-PS beads have remained intact but the majority of them had degraded into fine particles which were responsible for slow filtration rates. Therefore it can be concluded that sporopollenin is significantly more mechanically robust than a commercial resin.



(A)



(B)

Figure 4.6 Scanning Electron Micrographs of:
(A) Wang PS resin before stirring
(B) Wang PS resin after stirring for 1 day (24 hours)

4.3 FUNCTIONAL GROUP ANALYSIS

Various methods were used to investigate the surface functional groups of sporopollenin and their reactivity. This was undertaken to investigate potential means of attachment for applications in synthesis and discover what possible side reactions might take place with such synthetic applications.

4.3.1 Determination of the Iodine Value (IV) of Sporopollenin

A known amount of sporopollenin was reacted with a known excess of ICl (Wij's solution). The remaining ICl was then converted to I_2 by the addition of KI solution and the iodine was then titrated with standard $Na_2S_2O_3$ solution. The amount of ICl reacted with the sporopollenin was determined by comparison with a blank. The IV is defined as the amount of ICl expressed as grams of iodine absorbed by 100 g of product under the operating conditions used.[12] The IV could then be calculated (See Chapter 5, Section 5.2.9). The number of moles of ICl taken up per gram of sporopollenin was also calculated. The number of moles of ICl reacted with sporopollenin could therefore be directly related to the number of moles of double bond character in the sample.

Reaction Time between Sporopollenin and ICl	IV	Amount of ICl taken up (mmol/g)
2 hours	90 (2)	3.58 (2)
3 days	140-146 (2)	5.63 (2)
7 days	162-166 (3)	6.45 (3)

Table 4.7 Results of the Iodine Value determinations.
The Iodine values were rounded up to the nearest whole number.
The number in brackets represents the number of times the determination was carried out.
Sporopollenin sample 03 was used (See Table 4.1).

A high loading of ICl was observed and this is in accordance with the findings by Zetzsche and his co-workers[13] who found that after reaction of sporopollenin with bromine a bromine content of approximately 50% was found. This indicated the highly unsaturated nature of sporopollenin. These unsaturated groups could be used to achieve high loadings of functional groups *via* direct bromination or chlorination and then displacement with linkers used in solid phase synthesis or scavenging reagents.

4.3.2 Determination of the Acidity of Sporopollenin

A known weight of sporopollenin was reacted with a known of excess of NaOH solution and was left to react with the sporopollenin. Any acidic functionalities such as carboxylic acids and phenolic groups would react with the NaOH. The remaining excess NaOH was then titrated with HCl solution until the end-point was reached. The moles of NaOH consumed was then calculated and the results expressed in mmol of NaOH consumed per gram of sporopollenin which reflected the number of acidic groups on sporopollenin (See Chapter 5, Section 5.2.10). Table 4.8 shows the number of mmoles of NaOH consumed per gram of sporopollenin. Sporopollenin prepared using Method C was used for these determinations.

Reaction Time	mmol of NaOH consumed / g of sporopollenin
30 minutes	0.50-0.60 (4)
1 hour	0.52-0.76 (8)
2 hours	0.74-0.97 (9)
4 hours	1.30-1.38 (4)

Table 4.8 Results for the determination of the acidity of sporopollenin.
Sporopollenin sample 03 was used (See Table 4.1).
The numbers in brackets show how many times the determinations were carried out.

These results show that acidic groups present on sporopollenin are reactive and accessible to reagents such as bases.

4.3.3 Methods for the Detection of Aldehydes and Ketones in Sporopollenin

4.3.3.1 Reaction with Brady's Reagent

2,4-Dinitrophenylhydrazine was reacted with sporopollenin to establish if there were any aldehydes or ketones present on the surface (Chapter 5, Section 5.2.11.1). Any 2,4-dinitrophenylhydrazones formed during the reaction could be readily detected by IR spectroscopy. IR analysis of the derivatised sporopollenin showed that 2,4-dinitrophenylhydrazine had reacted and changes in the IR spectrum (Appendix 2, IR 13) compared to the underivatised sporopollenin had occurred. These changes are shown in Table 4.9.

Absorption Frequency (cm^{-1})	Functional Groups Present / Comments
~ 3440	Small peak on shoulder of –OH peak, possibly due to –NH group.
~ 1616	Strong, sharp peak indicates the presence of –C=N groups.
~ 1512 ~ 1335	Peaks in this region probably due to $-NO_2$ stretching.

Table 4.9 Important IR absorptions present in sporopollenin after derivatisation with 2,4-dinitrophenylhydrazine.

Elemental analysis of the derivatised sporopollenin showed that the nitrogen content had increased corresponding to attachment of 2,4-dinitrophenylhydrazine. Table 4.10 shows the loading of 2,4-dinitrophenylhydrazone, determined by elemental analysis, on sporopollenin after reaction with Brady's reagent for 30 minutes.

The loading of 2,4-dinitrophenylhydrazone is quite high and falls within the loading levels of those of commercial functionalised resins. These aldehyde/ketone groups could be exploited by derivatisation and used as further points of attachment in synthesis. Carbonyl functionalised resins are commercially available and have been used to immobilise amines.[11a,14] If these groups could potentially interfere in chemistry carried out on sporopollenin, these can be made inactive. One example of such reaction is the reduction of these aldehyde/ketone groups to the corresponding alcohol with $NaBH_4$ and then capping of these hydroxyls. This has been carried out on sporopollenin and is discussed in Section 4.4.6.

Sporopollenin Sample*	Method	Loading of 2,4-dinitrophenylhydrazone (mmol/g)
01	A	0.65 (1)
02	B	0.60 (1)
03	C	0.58-0.64 (2)
05	C	0.53-0.57 (2)
07	C	0.61-0.68 (2)

Table 4.10 Loading of 2,4-dinitrophenylhydrazone on sporopollenin after reaction with Brady's reagent after 30 minutes.
* See Table 4.1
The numbers in brackets indicates how many times the derivatisations were carried out.

4.3.3.2 Reaction of Sporopollenin with Fehling's Solution

Sporopollenin was heated with Fehling's solution for 5 minutes (Chapter 5, Section 5.2.11.2). After this time, the sporopollenin was collected by filtration. No colour change was observed under the reaction conditions. Since Fehling's solution tests for aldehydes and not ketones the reaction of sporopollenin with 2,4-dinitrophenylhydarzine suggests that sporopollenin contains ketones only and no aldehydes are present.

4.4 FUNCTIONALISATION OF SPOROPOLLENIN

4.4.1 Determination of the Number of Hydroxyls Available for Functionalisation

Since sporopollenin from *Lycopodium clavatum* is known to contain hydroxyl groups, (15 per C_{90} unit found by Zetzsche *et al*[15,16] and 15 per C_{90} unit found by Fawcett *et al*[17]), attempts were made to determine the number of hydroxyls on the surface using techniques used to routinely estimate the hydroxyl content of commercial resins[11b,14,18] (See Chapter 5, Section 5.2.7). 9-Fluorenylmethyl chloroformate **31** (Fmoc-Cl) is a common reagent for the protection of amino groups in peptide synthesis and is also commonly used for the quantitative analysis of these amino groups.[18,19] Fmoc-Cl also reacts with hydroxyls and has been adapted for use as an analytical method for the determination of hydroxyls on solid phase resins.[11b,14,18]

Attachment of Fmoc-Cl proceeds in the presence of pyridine in DCM. The electron withdrawing ring system of the Fmoc group makes the lone hydrogen on the β-carbon acid and susceptible to removal by weak bases.[19] Piperidine in DCM or DMF is routinely used.[11a-b,14,18] After H^+ extraction, Fmoc removal proceeds *via* a carbanion intermediate **32** to form 9-fluorenylmethene **33** which then reacts with excess piperidine to form the adduct, 9-*N*-(9-fluorenylmethyl)piperidine **34**. The absorbance of the adduct can then be measured at 301nm and is directly related to the number of hydroxyls on the support. This reaction scheme is shown in Figure 4.7.

The results of the Fmoc analyses are shown in Table 4.11 and the loading of hydroxyls on the sporopollenin are expressed in mmol/g. The Fmoc derivatisation was carried out in duplicate or triplicate for each sample, the cleavage reaction and absorbance measurements were carried out in triplicate for each separate derivatisation.

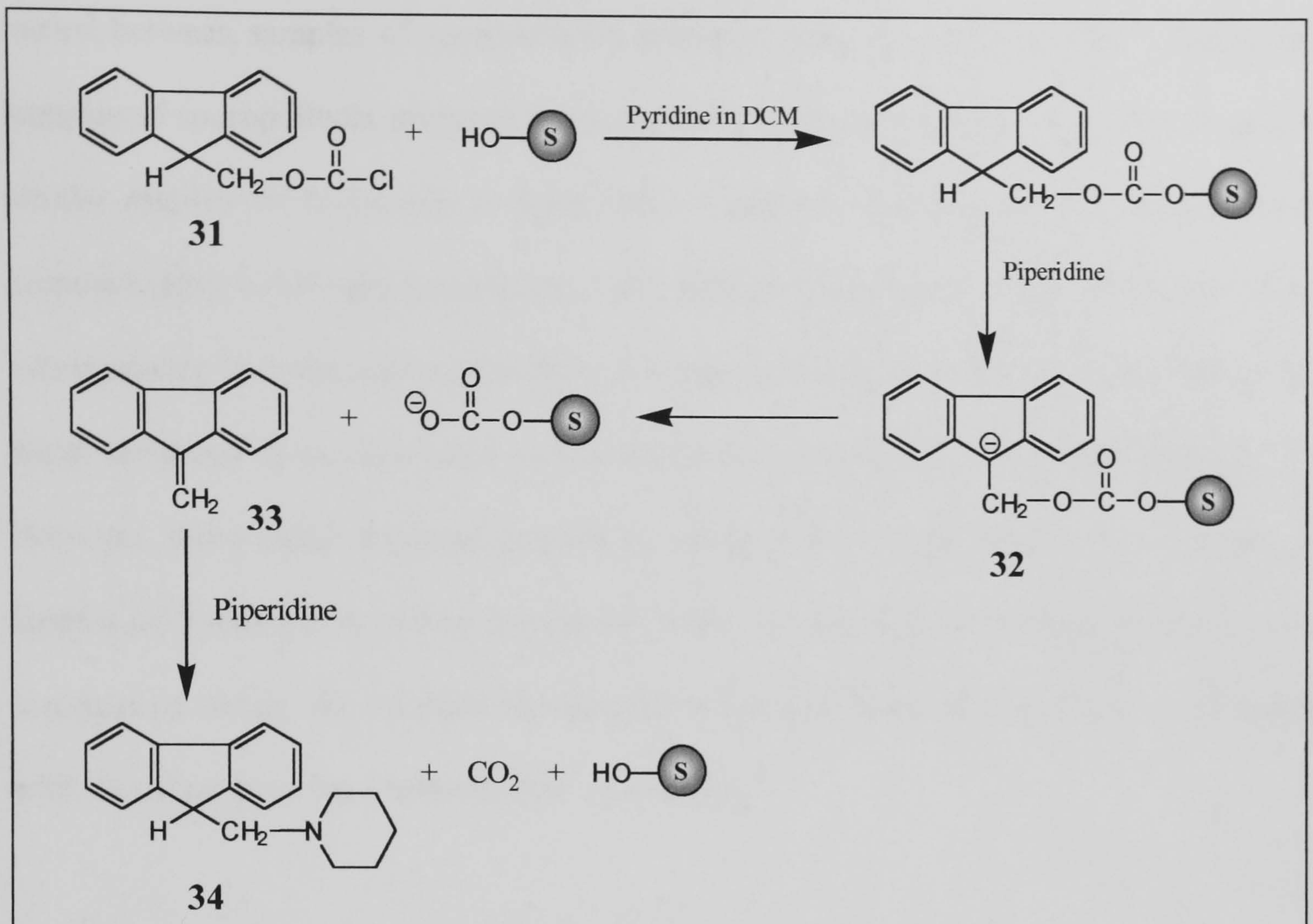


Figure 4.7 Fmoc Analysis.

Method of Isolation of Sporopollenin	Sample Number	Lycopodium Batch	Loading of Hydroxyls (mmol/g)
A	01	a	1.39
B	02	a	1.15
C	03	b	0.66
C	04	c	0.41
C	05	c	0.40
C	06	b	0.67
C	07	c	0.43
C	08	d	0.92

Table 4.11 The hydroxyl content of sporopollenin samples.

It was found that the number of hydroxyls available for derivatisation with Fmoc-Cl varied between samples of sporopollenin prepared using the same method. Again, the samples of sporopollenin prepared using the same batch of *L. clavatum* seemed to have a similar number of hydroxyls to each other compared with samples of sporopollenin prepared using a different starting batch of Lycopodium powder. There seemed to be an inconsistency in composition of starting Lycopodium powder which was responsible for slight variations in sporopollenin such as elemental analysis and number of hydroxyls. However, when using the same method of preparation of sporopollenin the variation in loading of hydroxyls is still in comparison with the variation of loading found in some commercial resins, for example Novabiochem's hydroxymethylpolystyrene is available with a loading anywhere between 0.6–1.6 mmol/g.[11a]

4.4.2 Direct Attachment of *N*-Fmoc Amino Acids to Sporopollenin

The proposed synthesis of aminoimidazoles and purines using sporopollenin involves the attachment of an *N*-Fmoc amino acid to a hydroxyl functionality on the sporopollenin. Despite the inconsistency in the natural hydroxyls present between the sporopollenin samples direct attachment of the protected amino acid to these hydroxyls were attempted (For methods, See Chapter 5, Section 5.6.3). The loading of amino acids onto sporopollenin prepared using Method C are shown in Table 4.12. Direct attachment of *N*-Fmoc amino acids gave low yields. Sporopollenin sample 03 received a double coupling of the amino acid using the three methods of attachment but this made no difference to the final loading. If the starting hydroxyl content of the sporopollenin was low as in samples 04 and 05 in Table 4.12 the loading of amino acid was very poor. Examination of the three methods used showed that the use of the coupling agent

PyBOP® lead to a higher attachment of amino acid in the sporopollenin samples. The ninhydrin assay (Chapter 5, Section 5.2.12) was performed in the samples of the amino acid derivatised sporopollenins to check that no cleavage of the Fmoc group had occurred. No cleavage of Fmoc group was observed and the unreactivity of these hydroxyls under the conditions attempted could have been due steric hindrance caused by the nearness of the hydroxyl groups to the sporopollenin surface.

Sporopollenin Sample*	Fmoc Amino Acid	Attachment Method	Loading	% of Hydroxyls Reacted
03	Glycine	PSA	0.17-0.25 (3) mean = 0.21	32
03	Glycine	DCC/HOBt	0.17-0.29 (3) mean = 0.25	38
03	Glycine	PyBOP®	0.34-0.41 (3) mean = 0.35	53
04	Alanine	PSA	0.05-0.09 (4) mean = 0.07	17
04	Alanine	DCC/HOBt	0.08-0.10 (3) mean = 0.09	22
04	Alanine	PyBOP®	0.14-0.19 (2) mean = 0.17	41
05	Alanine	PSA	0.05-0.06 (2) mean = 0.06	15
05	Alanine	DCC/HOBt	0.07-0.09 (3) mean = 0.08	20
05	Alanine	PyBOP®	0.11-0.19 (3) mean = 0.15	38

Table 4.12 Direct attachment of protected amino acids onto sporopollenin.
Functionalisation of samples 04 and 05 with *N*-Fmoc alanine was undertaken by co-workers.[27a,b] The number in brackets refers to the number of times the derivatisation was carried out, Fmoc cleavage was carried out in triplicate for each one.
Abbreviations for the reagents used can be found at the front of this thesis.
* See Table 4.1.

Sporopollenin sample 03, derivatised with Fmoc glycine, was exposed to TFA to determine the stability of the ester linkage between the sporopollenin and glycine. Fmoc analysis revealed that the majority of amino acid remained attached (See Figure 4.8).

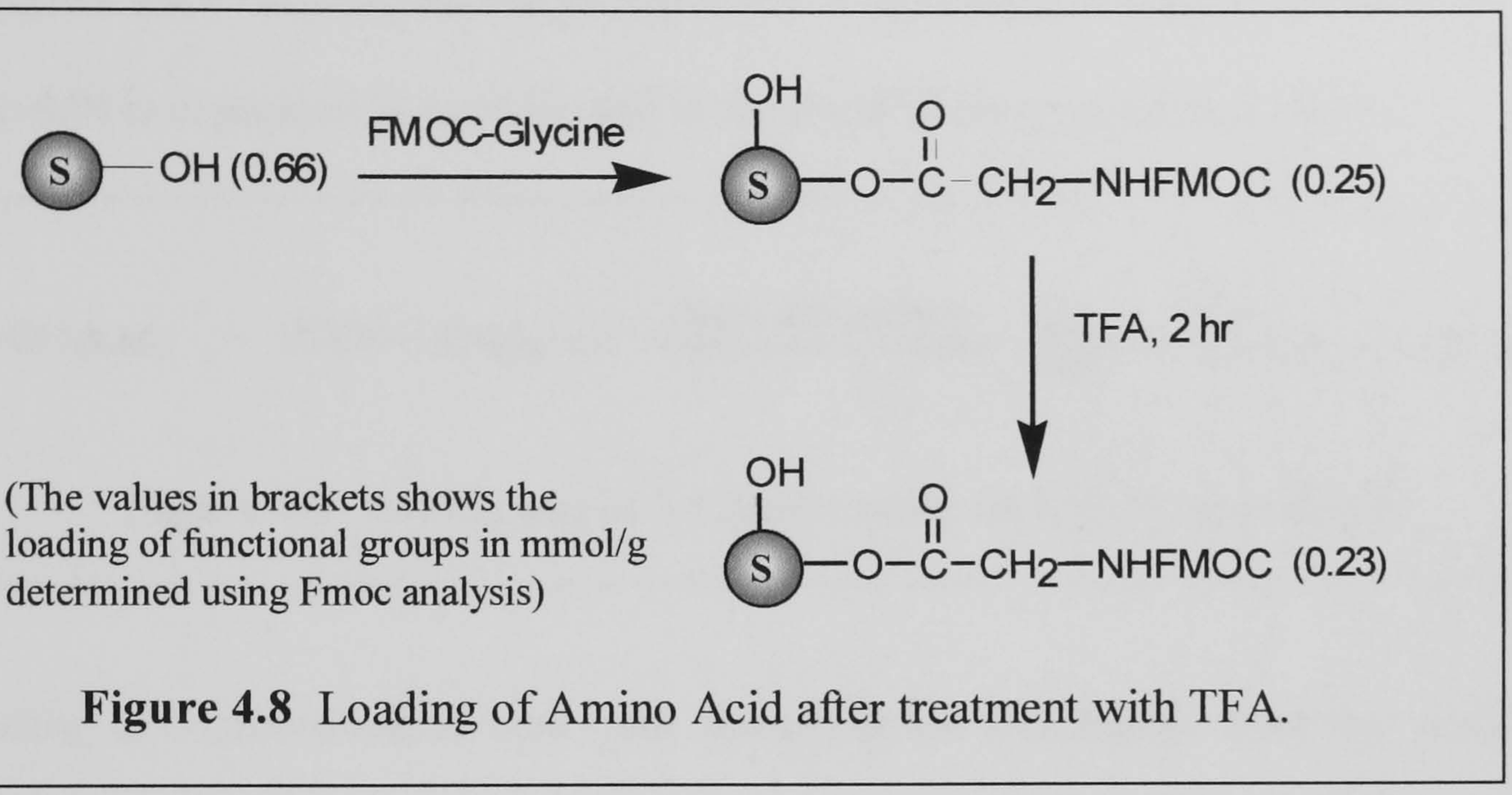


Figure 4.8 Loading of Amino Acid after treatment with TFA.

The stability of the ester formed to TFA is analogous to the ester formed upon reaction of carboxylic acids with hydroxymethylpolystyrene which requires HF or TFMSA for cleavage. Cleavage can also be affected with hydrogenolysis[20] or reduction with DIBAL[21] or $LiBH_4$[22] Cleavage of the sporopollenin amino acid esters could also be possible with these reagents but was not attempted because of the low loading of amino acid onto sporopollenin. Instead efforts were concentrated on finding a more suitable attachment method for the amino acid.

4.4.3 Functionalisation of Sporopollenin with 5-Chlorovaleric Acid

Attachment of other carboxylic acid containing groups other than amino acids were attempted. Sporopollenin prepared using Method C, Sample 03 was reacted with 5-chlorovaleric acid **35** using PyBop® as the coupling agent. (See Chapter 5, Section 5.6.2) Displacement of chloride with hydroxide could then produce a functionalised

hydroxyl support which could be more suitable for attachment of Fmoc-amino acids as the hydroxyl groups will be further away from the surface and more accessible. The attachment of 5-chlorovaleric acid was carried out in duplicate. Chloride analysis of these samples were then carried out in triplicate. The loading (values in brackets shown in Figure 4.9) is expressed in mmol/g and is the mean of these combined results.

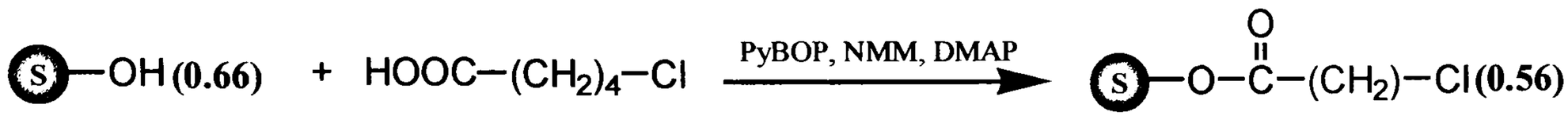


Figure 4.9 Attachment of 5-Chlorovaleric Acid to Sporopollenin.

The loading of 5-chlorovaleric acid onto sporopollenin was higher than that obtained using Fmoc amino acids. However, commercial hydroxy containing resins are available with loadings higher than these values obtained and it was decided that sporopollenin derivatised with 5-chlorovaleric acid was not a strong competitor for these resins.

4.4.4 Halogenation of Sporopollenin

Since there is evidence to suggest that sporopollenin contains various functional groups, namely alcohols, carboxylic acids and double bonds (Chapter 3, Section 3.3) per-chlorination of the sporopollenin surface was attempted. The purpose of these experiments was to attempt chlorination of these functional groups and to investigate if they could be selectively chlorinated. Chlorinated sporopollenin could provide a useful attachment for a range of linkers.

4.4.4.1 Chlorination with Phosphorus Pentachloride (PCl_5)

Phosphorus pentachloride can:

1 Convert alcohols to their corresponding alkyl halides[23,24]

2 Convert carboxylic acids to acid chlorides.[23]

3 Halogenate double bonds of alkenes.[23]

Chlorination of sporopollenin with PCl_5 was attempted using a quick and simple procedure adapted from a method used to prepared alkyl halides from tertiary alcohols.[24] This method used PCl_5 for short reaction periods at 0°C in an inert solvent using $CaCO_3$ as a buffer to absorb any acid generated. Aqueous insoluble $CaCO_3$ was replaced by K_2CO_3 which could be separated from the sporopollenin by washing with water.

This chlorination method was chosen because:

a) Mild conditions were used which would cause fewer changes to the sporopollenin.

b) The reaction times were relatively short. The time was extended from 5 minutes from a solution phase reaction to 10 minutes with sporopollenin.

c) The reaction work-up was simple and convenient.

The loading of chloride onto sporopollenin after reaction with PCl_5 was determined using the methods outlined in Chapter 5, Section 5.2.6. The results of these derivatisations are shown in Table 4.13. The amount of chloride displaced with pyridine was very low. Using a stronger base and longer reaction times resulted in higher loadings being observed. These results indicated the possibility of two types of chlorinated groups on the sporopollenin surface, one which is more reactive and easily removed with pyridine and the other which is not as reactive and requires stronger basic conditions for nucleophilic displacement. The loading of chloride was very similar to

the loadings of hydroxyls detected using Fmoc analysis. This was an indication that the hydroxyls had reacted with PCl_5.

Sporopollenin Sample*	Cleavage Conditions	Method of Chloride Analysis	Loading of Cl (mmol/g)
02	Pyridine, 2 hours	Volhard's Method A	0.16-0.20 (3)
02	2M NaOH, 2 hours	Volhard's Method B	0.50-0.68 (3)
		Gravimetric	0.54-0.70 (3)
02	2M NaOH, 8 hours	Volhard's Method B	1.11-1.15 (2)
		Gravimetric	1.00-1.20 (7)
03	2M NaOH, 8 hours	Volhard's Method B	0.62-0.67 (2)
		Gravimetric	0.60-0.81 (8)
04	2M NaOH, 8 hours	Gravimetric	0.35-0.40 (3)

Table 4.13 Results of chloride analyses after chlorination of sporopollenin with PCl_5. The numbers in brackets refers to the number of times the samples were chlorinated. The methods of chloride analysis can be seen in Chapter 5, Section 5.2.6.
* See Table 4.1

Modified Volhard's Method A

Briefly, the modified Volhard's method involved treatment of the hydrolysed chloride with an excess of $AgNO_3$ solution. The AgCl formed was coated in toluene and the iron (III) indicator was added. The excess $AgNO_3$ was then back titrated with NH_4SCN. When all the $AgNO_3$ had been consumed the end-point was indicated due to the formation of a red iron (III) complex with excess NH_4SCN. The determination of the end-point was very difficult to observe due to the presence of AgSCN and the toluene coated AgCl which were both precipitates making the solution cloudy. In addition only small amounts of NH_4SCN were added before the end-point was reached which meant that the solution was too concentrated and was likely to lead to inaccurate results.

Modified Volhard's Method B

The loading of chloride after displacement with pyridine was low. Sodium hydroxide was used instead of pyridine for the displacement of chloride from the sporopollenin to see if there were any differences in the amount of chloride released. Times of 2 hours and 8 hours were tried (Table 4.13). After hydrolysis with 2M NaOH, the filtrate was brown in colour which interfered with the detection of the end-point during the titration therefore the filtrate was decolourised with charcoal before addition of $AgNO_3$. Because the end-point of the titration was difficult to determine it was decided that the AgCl was to be removed before the titration. This AgCl precipitate was collected by filtration and could be used for gravimetric analysis. A 0.02M NH_4SCN solution was used instead of a 0.1M solution. Consistency between the results of titrimetric and gravimetric were observed. Gravimetric was found to be the favoured method due to its simplicity and efficiency. The infrared spectrum of sporopollenin chlorinated with PCl_5 (Appendix 1, IR 14a) indicates that phosphorus could be present in the samples.

Table 4.14 Shows the important IR absorptions which have changed or appeared with respect to the underivatised sporopollenin.

Carman and Shaw[24] showed that one of their alcohols, cis-terpin, when reacted with PCl_5 contained phosphorus. The infrared spectrum of the alkyl halide showed strong absorptions at 1300-1250 cm^{-1} and 1050-1000 cm^{-1} (indicative of –PO–O–C groups). The workers suggested that phosphodiester bridging between the two hydroxyl groups had occurred. This prompted the need to assay for phosphorus on the PCl_5 treated sporopollenin.

Absorption Frequency (cm^{-1})	Functional Groups Present / Comments
~ 3400	This peak has been reduced and corresponds to a reduction in the number of hydroxyls present. This was a good indication that –OH groups had been converted into chlorides.
~ 1200	This broad, medium intensity peak is absent in the underivatised sporopollenin and could be due to –P=O and –P–O–C bonds.
~ 1000	This strong, sharp peak could also be due to –PO–O–C groups.
~ 490	This absorption is absent in the underivatised sporopollenin.

Table 4.14 IR absorptions of Sporopollenin after derivatisation with PCl_5.

Phosphorus Analysis

Reaction of phosphate with ammonium molybdate gives a yellow precipitate of ammonium phosphomolybdate $(NH_4)_3[PMo_{12}O_{40}]$. When this is reacted with a reducing agent containing p-methylaminophenol sulfate, an intense blue colour develops due to the reduction of molybdate in the precipitate. Any uncombined ammonium molybdate remains unaffected. A sample of chlorinated (PCl_5) sporopollenin was heated with 2M NaOH and the above procedure carried out. The solution turned and intense blue colour indicating the presence of phosphorus.[29] Elemental analysis of sporopollenin sample 02 chlorinated with PCl_5 showed a phosphorus content of 1.14 mmol/g and a chloride content of 2.23 mmol/g. Only half of this chloride could be detected upon base hydrolysis for 8 hours at 100°C.

It was unsure at this stage if this phosphorus could be a problem during further derivatisation of sporopollenin or if it would interfere with any synthesis carried out on

sporopollenin. If necessary any phosphate esters formed could be hydrolysed with base or a phosphorylase enzyme.

4.4.4.2 Chlorination using Thionyl Chloride ($SOCl_2$)

A solution phase method of preparing alkyl halides from alcohols was adapted to solid phase and was carried out on sporopollenin (Chapter5, Section 5.6.1).[26] The results of triplicate gravimetric analysis were between 0.72-0.78 mmol/g. Elemental analysis showed that sulfur (1.47 mmol/g) had been incorporated into the sporopollenin structure. A loading of 0.85 mmol/g of Cl per gram of sporopollenin was also found using elemental analysis, slightly higher than that found using displacement with base. The IR spectrum (Appendix 1, IR 14b) after chlorination with $SOCl_2$ shows additional absorptions at 878 and 730 cm^{-1} compared to the underivatised spectrum. These absorptions are probably due to $-CH_2$-Cl groups.

Sporopollenin chlorinated with PCl_5 and $SOCl_2$ produced chloro derivatives of sporopollenin. This chlorine groups could be displaced by hydroxide which indicates the potential for the attachment of linkers by nucleophilic displacement.

4.4.5 Capping of the Naturally Occurring Hydroxyls on Sporopollenin

Because the reaction of sporopollenin with PCl_5 and $SOCl_2$ introduced a large amount of phosphorus and sulfur respectively attempts to try different methods of functionalisation, other than derivatisation of the naturally occurring hydroxyls, were tried. In order to remove these hydroxyls from the surface studies were carried out to find a suitable method of protection of these groups. Removal of these hydroxyls would prevent them

interfering with any subsequent reaction and Fmoc analysis of any hydroxy functionalised linkers which may be attached in the future.

4.4.5.1 Acetylation and Acylation of Sporopollenin

Various methods of acetylation were attempted (Chapter 5, Section 5.6.4.1). Acetylation has already been carried out by other workers.[1,16,17] Method A involved refluxing acetic anhydride and Methods B to D involved the used of a catalytic amount of DMAP. Fmoc analysis of the acetylated sporopollenins showed that not all the hydroxyls had been acetylated under the conditions used. Table 4.15 shows the results of the acetylation reactions. The loading of unreacted hydroxyls were determined by Fmoc analysis and the values shown are the average loading of three separate acetylation reactions. Fmoc analysis was carried out in triplicate for each separate acetylation. The infrared spectra of these acetylated sporopollenin derivatives were similar and indicated the presence of unreacted hydroxyls. IR 15 shows a) sporopollenin acetylated with acetyl chloride and b) underivatised sporopollenin (Appendix 1).

Acylation of sporopollenin has been carried out using benzoyl chloride and trimethyl acetyl chloride by a member of the group and similar results were obtained.[27d] Acylation with benzoyl chloride on a 0.1g sporopollenin scale resulted in 0.06 mmol/g of hydroxyls unprotected and on a 1g scale, 0.12 mmol/g of hydroxyls were unprotected. Similarly using trimethyl acetyl chloride on a 0.1g scale 0.04 mmol/g of hydroxyls remained unprotected and a value of 0.12 mmol/g of hydroxyls were obtained for a 1g scale reaction.

Sporopollenin Sample*	Method of Acylation	Hydroxyls Unreacted (mmol/g)	No of Hydroxyls Available before Acetylation (mmol/g)	% Hydroxyls Acetylated
02	Acetic anhydride Method A	0.26	1.15	77
	Acetic Anhydride, Method B	0.24	1.15	79
	Acetic anhydride, Method C	0.20	1.15	83
	Acetic anhydride, Method D	0.18	1.15	84
05	Acetyl chloride, Method A, 0.1g scale	0.08	0.40	80
	Acetyl chloride, Method A, 1g scale	0.24	0.40	40
	Acetyl chloride, Method B, 0.1g scale	0.06	0.40	85
	Acetyl chloride, Method B, 1g scale	0.10	0.40	75
	Acetyl chloride, Method C, 1g scale	0.09	0.40	78
07[1]	Acetyl chloride, Method B, 0.1g scale	0.06	0.43	86
	Acetyl; chloride, Method B, 1g scale	0.12	0.43	72

Table 4.15 Acetylation of Sporopollenin.
Sporopollenin sample 07 was acetylated by a co-worker from the Lycopodium group.[27d]
The procedures for acetylation can be found in Chapter 5, Section 5.6.4.1
* See Table 4.1.

Acetylation with acetic anhydride was not as efficient as capping of the hydroxyls with acetyl chloride. Acetylation with 6 equivalents of acetyl chloride was more efficient than with 3 equivalents. The concentration of hydroxyls underivatised in the samples were between 0.04-0.08 mmol/g using a 0.1g scale of sporopollenin. Large scale acetylation of 1g was not as efficient as the smaller scale. This problem was largely overcome using

6 equivalents of acetyl chloride or heating the reaction mixture. Double coupling of the sporopollenin sample 05 with 6 equivalents of acetyl chloride brought the number of free hydroxyls down form 0.10 mmol/g to 0.06 mmol/g. These results showed that there was a base level of unreacted hydroxyls of 0.06 mmol/g for most of the reagents tested and using more concentrated reagents, heat, or double coupling did not reduce the number of available hydroxyls any further.

It was therefore concluded that these underivatised hydroxyls were effectively unreactive towards modification or that this low value could have been due to residual Fmoc-Cl adsorbed onto the sporopollenin in some way.

4.4.6 Reduction of Sporopollenin

There is evidence, both literature (Chapter 3, Section 3.3) and research mentioned in this thesis (Sections 4.3.2 and 4.3.3), to support the existence of carboxylic acids and ketones on sporopollenin. In order to obtain uniformity of functional groups on the surface, reduction of the sporopollenin was investigated. Reduction of the surface would also prevent any side or competing reactions during any further synthesis. Therefore it was decided to attempt the reduction of any acids or ketone carbonyls present on the surface with $LiAlH_4$ and $NaBH_4$ (Chapter 5, Section 5.5). After reduction using these two reagents the hydroxyl content of the sporopollenin had increased. The loading of hydroxyls after reduction can be seen in Table 4.16.

Sporopollenin Sample*	Reducing Agent	Loading Before Reduction (mmol/g)	Loading after Reduction (mmol/g)	% Increase in Hydroxyls
03	$LiAlH_4$	0.66	1.22 (3)	46
05	$NaBH_4$	0.40	0.91 (4)	42

Table 4.16 Loading of hydroxyls of sporopollenin before and after reduction.
The values in brackets refer to the number of times the reduction was carried out.
* See Table 4.1.

The IR spectra (Appendix 1, IR 16 and 17) show sporopollenin after reduction with $LiAlH_4$ (IR 16) and $NaBH_4$ (IR 17). It is evident from the IR spectra that an increase in absorption at around 3500 cm^{-1} corresponds to an increase in hydroxyl content. In conjunction the carbonyl absorption at around 1700 cm^{-1} was reduced in intensity compared with the underivatised sporopollenin. This corresponds to reduction of carbonyl containing groups to hydroxyls. Although the sporopollenin reduced using $LiAlH_4$ and $NaBH_4$ were different samples, they both had a similar percentage increase in hydroxyls. Using $LiAlH_4$ 0.56 mmol/g of extra hydroxyls were produced and with $NaBH_4$ 0.51 mmol/g extra hydroxyls were generated. This also corresponded to the loading of 2,4-dinitrophenylhydrazine on the sporopollenin samples (See Section 4.3.3, Table 4.10). Because similar results were obtained using $LiAlH_4$ and $NaBH_4$ it was suspected that the majority of new hydroxyls were due to reduction of ketones on the surface and not carboxylic acids since $NaBH_4$ cannot normally reduce carboxylic acids. Attempts to chlorinate these extra hydroxyl groups with PCl_5 proved only partially successful. The results of which are shown in Table 4.17.

Sporopollenin Sample*	Loading of hydroxyls after reduction (mmol/g)	Loading of Chloride after reaction with PCl_5 (mmol/g)	% Hydroxyls chlorinated
03	1.28	0.93	73
03	1.21	0.78	65
03	1.18	0.79	67

Table 4.17 Results of experiments after reduction followed by chlorination (PCl_5).
* See Table 4.1.

4.4.6.1 Protection of the Hydroxyls on Sporopollenin after Reduction

Sporopollenin after reduction was acetylated and methylated to determine if these extra hydroxyls could be protected. Sporopollenin reduced using $NaBH_4$ was acetylated using acetyl chloride, Method B (Chapter 5, Section 5.6.4.1) and methylated using methyl iodide and dimethylsulfate (Chapter 5, Section 5.6.5). Fmoc analysis was used to determine the number of hydroxyls which had been acetylated and methylated. The results of these determinations are shown in Table 4.18.

Sporopollenin Sample*	Method of Protection	Loading of Hydroxyls after Reduction	Loading of unprotected Hydroxyls
05	Acetyl chloride Method B	0.06 (3)	0.91
05	Methylation using Methyl Iodide	0.53 (2)	0.91
05	Methylation using Dimethyl sulfate (Method A)	0.41(2)	0.91
05	Methylation using Dimethyl sulfate (Method B)	0.33 (3)	0.91

Table 4.18 A comparison of the protection methods for hydroxyls on sporopollenin reduced using $NaBH_4$
* See Table 4.1.

From the results shown in Table 4.18 it can be seen that protection of the hydroxyls with acetyl chloride is the most efficient method. Again, a base hydroxyl value of 0.06 mmol/g was found. Methylation with both methyl iodide and dimethylsulfate was inefficient. It is proposed that the addition of a phase transfer catalyst such as tetrabutylammonium iodide may enhance the methylation reaction.[28]

From these acetylation and methylation studies it was concluded that the best method of protection of hydroxyls on the sporopollenin surface was one using acetyl chloride.

4.4.7 Chloromethylation of Sporopollenin

Chloromethylation of aromatic groups in polystyrene supports is a common method for introducing reactive species or linkers onto the support.[18,29] Chloromethylation of sporopollenin was tried because evidence provided in the literature suggests the presence of aromatic groups in sporopollenin (Chapter 3, Section 3.3). Additionally, previous studies undertaken by Mackenzie and Shaw found that sporopollenin could be chloromethylated at a level of around 1 mmol/g.[30] (See Chapter 3, Section 3.4.1) These chloromethyl groups would be ideal for displacement with a range of linkers. It was proposed that a functionalised sporopollenin analogous to Wang PS resin should be prepared. The synthesis of this functionalised sporopollenin can be seen in Figure 4.10. In order to obtain uniformity of the surface and selectivity the sporopollenin would be reduced. Chloromethylaton would then be carried out to give the functionalised sporopollenin **35** (Figure 4.10). The linker **36** could then to be attached in the aldehyde form so that selectivity during the capping of hydroxyls could be attained. Additionally, attachment of 4-hydroxybenzaldehyde would provide a means of monitoring of the reaction with Dansyl hydrazine or 2,4-dinitrophenyl hydrazine. The 4-

hydroxybenzaldehyde could then be selectively reduced to the alcohol with $NaBH_4$ to produce the hydroxybenzyl functionalised sporopollenin **37**. Reagents which have been used for chloromethylation of polystyrene supports include stannic chloride or zinc chloride with chloromethyl methyl ether.[18] The main disadvantage of this method is that chloromethyl methyl ether is carcinogenic and must be handled with care. Chloromethylation of polystyrene has also been carried out using dimethoxymethane, thionyl chloride and zinc chloride as a catalyst.[214] The reagents used

in this procedure are less harmful and chloromethylation of sporopollenin was carried out using this method.

Both non-reduced and reduced sporopollenin samples were chloromethylated using the methods outlined in Chapter 5, Section 5.7. The number of chloromethyl sites were determined by the amount of chloride released upon treatment with base for 8 or 48 hours. The results of these chloromethylations are shown in Table 4.19, page 140.

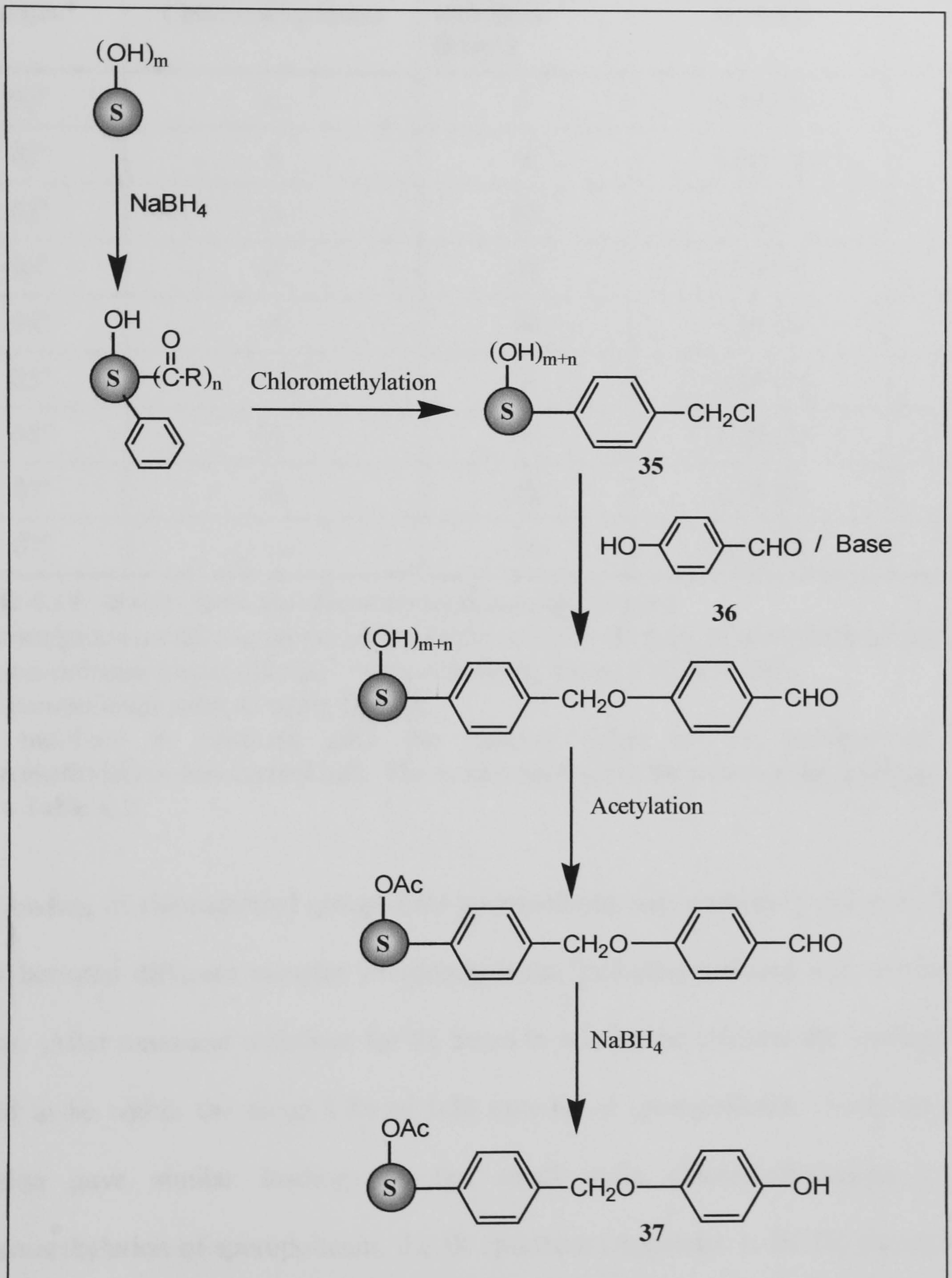


Figure 4.10 Synthesis of 4-hydroxybenzyl alcohol derivatised Sporopollenin *via* chloromethylation.

Sporopollenin Sample*	Method of Chloromethylation	Cleavage time with Base (hours)	Loading of chloride (mmol/g)	Scale (g)
03[a]	A	8	0.83 (2)	1
03[a]	A	8	0.89 (2)	4
03[a]	A	48	1.28 (1)	4
03[a]	A	48	1.16 (3)	1
05[a]	A	48	1.09 (3)	1
05[a]	B	8	0.66 (3)	1
05[b]	A	48	1.10 (3)	1
07[a]	A	48	1.08 (2)	1
07[c]	A	48	1.01 (1)	1

Table 4.19 Results form the chloromethylation experiments.
Superscript letters after sporopollenin sample refers to the type of sporopollenin used:
[a] = Non-reduced sporopollenin, [b] = Sporopollenin reduced with $NaBH_4$,
[c] = Sporopollenin reduced using $LiAlH_4$.
The numbers in brackets after the loading refers to the number of times chloromethylation was carried out. The values shown are the mean of the loadings.
* See Table 4.1

The loading of chloromethyl groups onto sporopollenin was consistent and reproducible even between different samples of sporopollenin including reduced and non-reduced forms. After treatment with base for 48 hours to release the chlorine the loadings were found to be within the range 1.01 to 1.28 mmol/g of sporopollenin. Scale up of the reaction gave similar loadings to the small scale chloromethylations. After chloromethylation of sporopollenin, the IR spectrum (Appendix 1, IR 18) shows a new absorption at 1107 cm^{-1} probably due to the presence of $-CH_2Cl$ groups.

Attempts to displace the chloride of the chloromethyl groups with the 4-hydroxybenzaldehyde linker resulted in low attachment using conditions normally used

to attach 4-hydroxybenzyl alcohol to chloromethylated polystyrene.[31] Table 4.20 shows the conditions used to attach 4-hydroxybenzaldehyde to chloromethylated sporopollenin.

Sporopollenin Sample*	Base and Solvent used	Reaction time (hours)	Initial chloromethyl loading (mmol/g)	Loading of Linker (mmol/g)	Yield (%)
03	K_2CO_3,DMA	8	1.16	0.30	26
03	NaOMe, DMA	8	1.16	0.32	28
03	NaOMe, DMA	48	1.16	0.43	37
03	K_2CO_3, MeOH	48	1.28	0.42	33
03	NaOMe, MeOH	48	1.28	0.40	31
07	NaH, CH_3CN	24	1.11	0.28	25
07	NaH, CH_3CN	48	1.11	0.37	33

Table 4.20 Reaction of 4-hydroxybenzaldehyde linker with chloromethylated sporopollenin.
* See Table 4.1.

Results of the displacement of chloride with 4-hydroxybenzaldehyde showed that no more than 37% of the linker had attached. This indicated that most the chloromethyl groups were unreactive or inaccessible to reagents and conditions normally used to chloromethylate aromatic rings. The chloromethylation reaction may have substituted chloromethyl groups onto other species other than aromatic groups of the sporopollenin and different types of chloromethyl groups may be present. Because sporopollenin contains double bonds there is a strong possibility that these could have been chloromethylated resulting in aliphatic chlorides which are less reactive towards further modification. This has been observed with PS-DVB copolymers which contain unreacted vinyl groups. Chloromethylation of these double bonds produces aliphatic chlorine groups which are difficult to modify.[29] Figure 4.11 shows the chloromethylation of aromatic groups and double bonds.

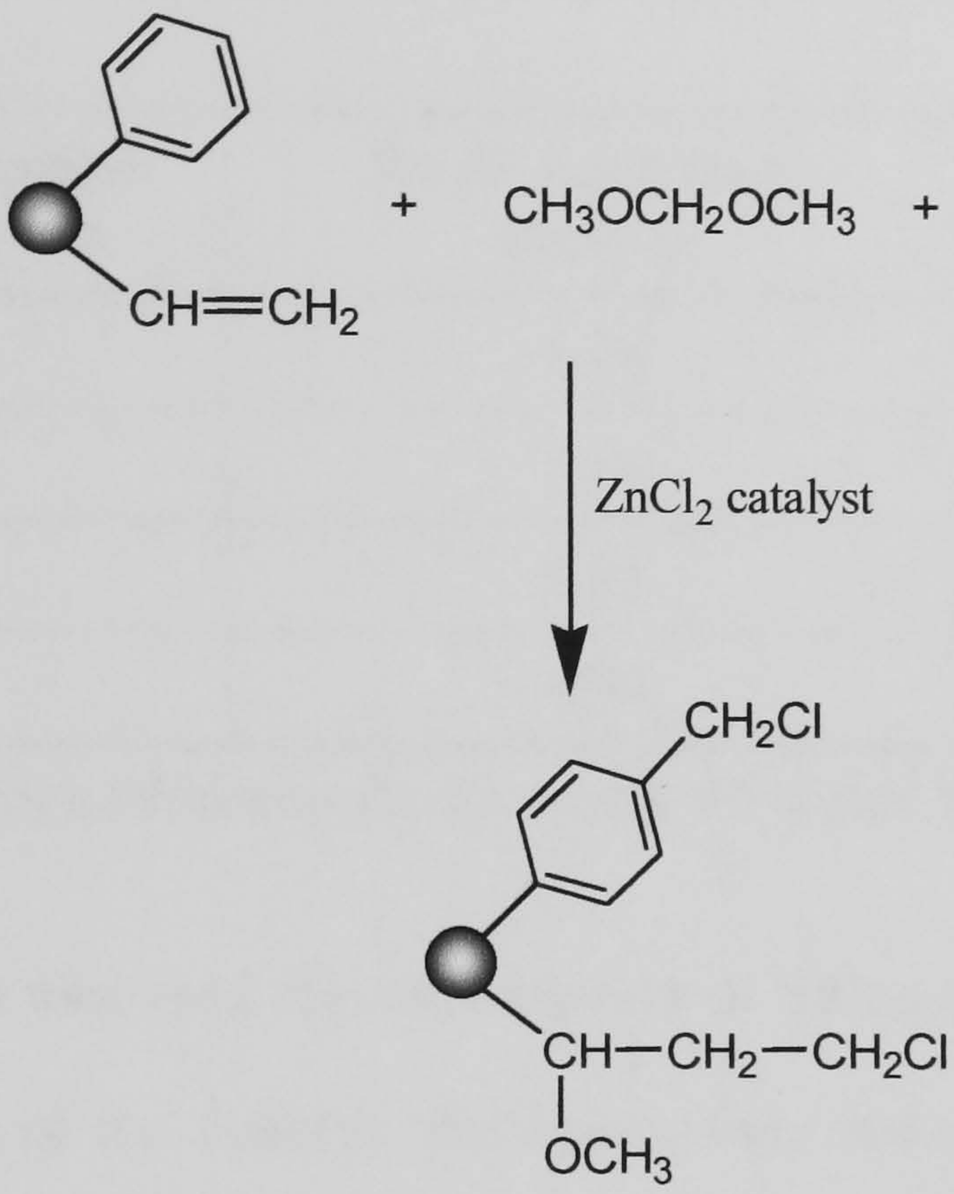


Figure 4.11 Chloromethylation of aromatic and vinylic groups.[29]

Because of the unreactivity of some of the chloromethyl groups efforts were turned to amination of sporopollenin instead of trying new methods of substituting these unreactive chloromethyl groups.

4.4.8 Amination of Sporopollenin

Amination of sporopollenin has been carried out by previous workers[32] with attachment of 1,3-diaminopropane achieving a loading of 1.6 mmol of base/g determined using the picric acid assay. Amination with diamines could be used for the attachment of linkers for solid phase synthesis or drugs for use in drug delivery.

4.4.8.1 Amination of Sporopollenin with 1,3-diaminopropane

The following results (Table 4.2.1) were found after amination of sporopollenin with 1,3-diaminopropane. (See Chapter 5, Section 5.9 for experimental details)

Method of Amination	Picric Acid Assay mmol/g	Elemental analysis mmol/g
A	1.10	2.28
B	1.18	1.30
B	1.05	1.51
B	0.99	1.70

Table 4.21 Amination of sporopollenin sample 03 with 1,3-diaminopropane.

Elemental analysis measured the total amount of nitrogen present in the sample. To obtain the loading of the diamine, the values were halved. These halved values are shown in Table 4.2.1. The picric acid assay measures the amount of basic groups on the sporopollenin after amination. The values in the Table are the loading of base taking into account the blank reading from the underivatised sporopollenin. There is a slight variation between the literature value (1.6 mmol/g) and the results reported in this thesis. Adamson *et al*[32] provided evidence to support the theory that the picric acid was reacting at both ends of the diamine. More recent studies by co-workers[27d,e] showed that sporopollenin after reaction with ammonia or mono-amines did not have a basic group attached to the sporopollenin. This was determined by treatment with HCl followed by chloride analysis. The results from the picric acid assay and elemental analysis vary considerably. Explanations for this are that the $-NH_2$ groups were not fully reacted with the picric acid due to steric hindrance or that cross-linking of the diamine was occurring. Also the lack of basicity and hence poor reactivity to picric acid might be an indication of an amide linkage being formed between the primary amide and sporopollenin.

The problem of cross-linking, which reduces the amount of free amine available, can be overcome by reacting sporopollenin with a mono-protected diamine.

The stability of the linkage between the diamine and the sporopollenin was assessed. The aminated sporopollenin was exposed to various acidic and basic conditions including TFA which is commonly used to cleavage products from solid supports.

The values of the loading of amine before and after treatment are shown in Table 4.22.

Conditions	Loading after treatment (mmol/g)	% Amine still attached
2M NaOH, RT, 4 days	1.97 (2)	86
2M NaOH, Reflux, 24 hours	1.67 (2)	73
2M HCl, RT, 4 days	2.01 (2)	88
2M HCl, Reflux, 24 hours	1.70 (2)	75
50% TFA, RT, 24 hours	1.97 (2)	86
50% TFA, RT, 48 hours	1.69 (2)	74
TFA, RT, 48 hours	1.70 (2)	75

Table 4.22 Stability of sporopollenin aminated with 1,3-diaminopropane. Sporopollenin sample 03 with a loading of 2.28 mmol/g by elemental analysis was used. The loading was determined by triplicate elemental analysis, the stability tests were carried out in duplicate and values shown is the average loading of these tests.

Sporopollenin aminated with 1,3-diaminopropane was shown to have good stability even after exposure to harsh and prolonged treatments.

4.4.8.2 Amination with n-Butylamine

Firstly to give a more clearer indication of loading of amine the sporopollenin was functionalised with n-butylamine. (See Chapter 5, Section 5.9.2) This only has one $-NH_2$ group therefore will not be able to cross-link to the sporopollenin. This would then provide information into the consistency and reproducibility of the amination

reaction between different sporopollenin samples. The loading of sporopollenin aminated with butylamine was determined by elemental analysis and the results are outlined in Table 4.23.

Sporopollenin Sample*	Loading (mmol/g)
03	1.30 (1)
03	1.21-1.59 (2)[27d]
04	1.84 (1)
04	1.14-1.80 (3)[27e]
04	1.50-1.98 (2)[27d]
05	1.00-1.22 (3)
06	1.26-1.56 (3)
07	1.52-1.78 (3)
08	1.74-1.82 (3)
09	1.90-2.11 (3)[27d]

Table 4.23 Amination of sporopollenin with n-butylamine using Method A.
* Table 4.1.

The loading of benzylamine using Method A onto sporopollenin was high and was comparable to the loadings of functionalised commercial resins. The loading of butylamine between the sporopollenin samples was reasonably consistent although not as consistent as the loading of chloromethyl groups after chloromethylation.

Amination of sporopollenin sample 05 with n-butylamine using Method B involved the use of DCC/DMAP at room temperature. The loading of amine found after elemental analysis was 0.55 mmol/g. The loading of n-butylamine after reaction at room temperature without the addition of DCC and DMAP was found to be 0.50 mmol/g. There was little difference between these values and it appeared that DCC did not

activate any carboxylic acids present on the sporopollenin at room temperature. The stability of the butylamine sporopollenin to TFA was similar to that of the 1,3-diaminopropyl sporopollenin. Using 50% TFA, 70% of the amine remained attached after 24 hours.

4.4.8.3 Amination of Sporopollenin with Mono-*N*-Boc-1,6-hexanediamine

In order to carry out the proposed imidazole and purine synthesis on the sporopollenin support. Amination followed by attachment of a linker to which the amino acid could be attached was proposed (Figure 4.12). Firstly, attachment of mono-*N*-Boc-1,6-hexanediamine **38** is required followed by acetylation of the residual hydroxyls. Removal of the Boc protecting group using TFA would then release the free amine on the sporopollenin **39**. Attachment of 4-hydroxymethylphenoxyacetic acid as its 2,4,5-trichlorophenyl ester **40** would then yield the hydroxy functionalised sporopollenin support **41**.

Sporopollenin was derivatised with mono-*N*-Boc hexanediamine on a 0.1g, 0.7g and 1g scale. The loadings were determined by elemental analysis; the results of which are shown in Table 4.24.

Amount of sporopollenin used in the reaction (g)	Loading (mmol/g)
0.1	1.19-1.27 (3)
0.1	0.92-0.93 (3)[27d]
0.7	0.39-0.48 (3)
1	0.38 (1)

Table 4.24 Results for the amination of sporopollenin sample 05 with mono-*N*-Boc protected 1,6-hexanediamine.

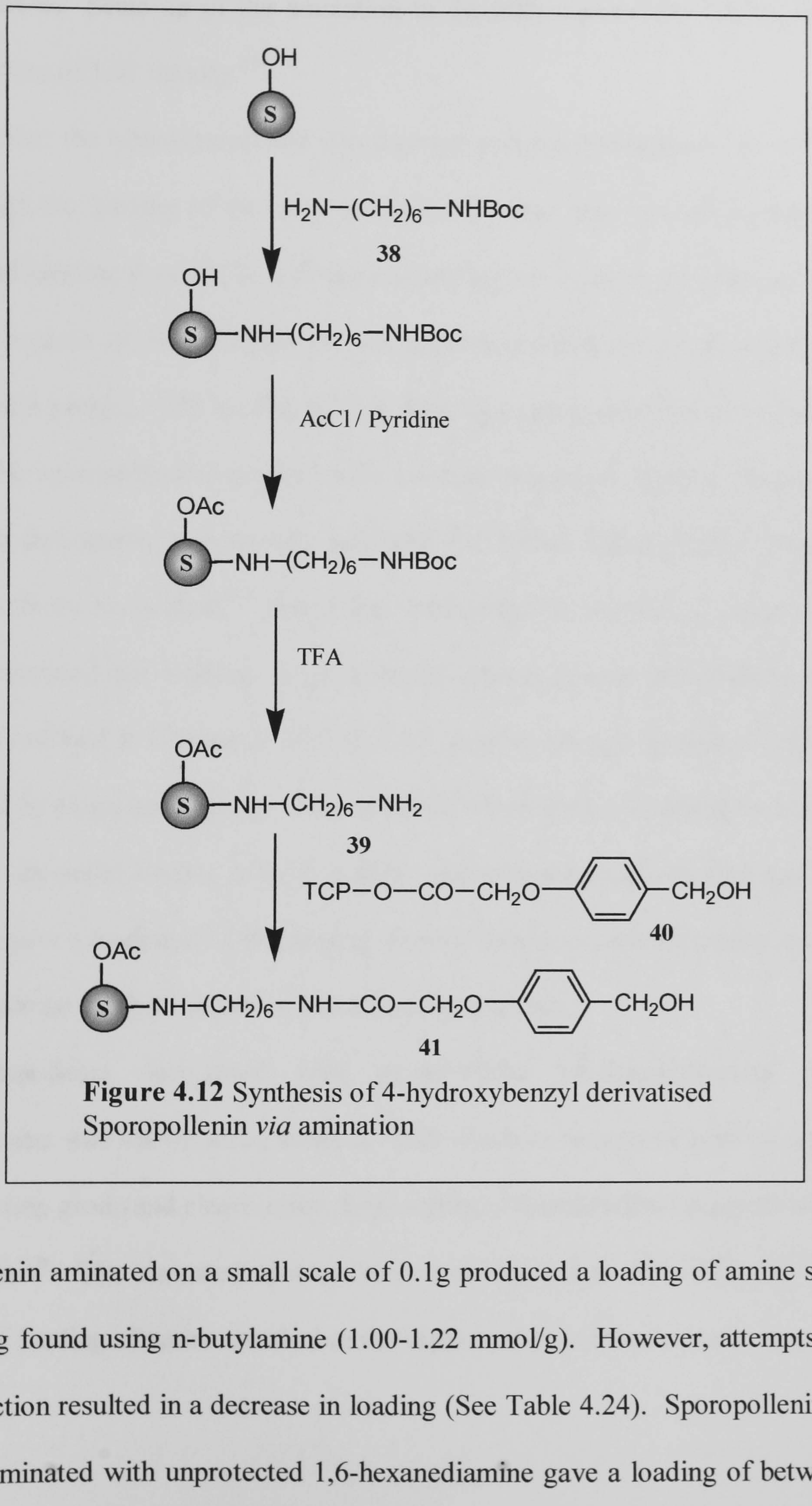


Figure 4.12 Synthesis of 4-hydroxybenzyl derivatised Sporopollenin *via* amination

Sporopollenin aminated on a small scale of 0.1g produced a loading of amine similar to the loading found using n-butylamine (1.00-1.22 mmol/g). However, attempts to scale up the reaction resulted in a decrease in loading (See Table 4.24). Sporopollenin sample 05 when aminated with unprotected 1,6-hexanediamine gave a loading of between 1.70 to 2.00 mmol/g, slightly higher than the loading obtained with mono-*N*-Boc 1,6-

hexanediamine. Scale up of the amination to 1g with unprotected 1,6-hexanediamine gave a loading of 1.01 mmolg.[27d]

It seemed that the t-butoxycarbonyl (Boc) group prevented attachment of the diamine. Even though the loading of the mono-protected diamine was reduced compared to the unprotected diamine it would be still more advantageous to use to the protected diamine. This was because of the potential for the unprotected diamine to cross-link onto the sporopollenin surface. The loading of 1.01 mmol/g was measured by elemental analysis only for the sporopollenin aminated with 1,6-diaminohexane, hence it was not known how much free amine was actually available for further modification. Interestingly, recent results by co-workers[27d] found that increasing the amount of amine during the reaction increased the loading. Sporopollenin aminated with benzylamine using the conditions outlined in Chapter 3, Section 5.9.3 gave an average loading of 1.24 mmol/g determined by elemental analysis. Increasing the concentration of amine by 10 fold gave an average increased loading of 2.40 mmol/g, and increasing the concentration of amine by 20 fold gave a loading of 3.38 mmol/g. Further washings were necessary to ensure all unreacted amine had been removed from the sporopollenin.

The sporopollenin derivatised with mono-*N*-Boc 1,6-hexanediamine and 1,6-hexanediamine was shown to be stable to TFA which is commonly used to remove the Boc protecting group and cleave esters from activated functionalised supports such as the Wang resin.[11a] The aminated sporopollenin was exposed to 50% TFA for 2 and 24 hours. The loading of amine attached to the support after treatment can be seen in Table 4.25.

Type of 1,6-hexanediamine	Scale (g)	Loading before TFA exposure (mmol/g)	Loading after treatment with TFA (mmol/g)			
			2 hours	% still attached	24 hours	% still attached
Unprotected	1	1.01	0.87	86	0.76	75
Protected	1	0.38	0.32	84	0.31	82
Protected	0.7	0.48	0.45	94	0.36	75

Table 4.25 Stability of sporopollenin aminated with 1,6-hexanediamine and mono-*N*-Boc 1,6-hexanediamine
Stability tests were carried out in triplicate.
The loading was determined by triplicate elemental analysis.

The stability of the sporopollenin aminated with protected and unprotected 1,6-hexanediamine to TFA showed comparable stability to sporopollenin aminated with 1,3-diaminopropane and n-butylamine. Even after 24 hours, no less than 75% of the diamine was removed.

In order to continue with synthesis of the hydroxybenzyl functionalised sporopollenin support **41** it was necessary to protect the hydroxyls of the mono-*N*-Boc 1,6-hexanediamine functionalised sporopollenin before cleavage of the Boc protecting group with TFA. It was necessary to remove these hydroxyls to allow ease of analysis of the attachment of the linker **40** and to prevent them from reacting in further synthesis. Removal of the Boc protecting group was carried out using 50% TFA in DCM in order to assess the stability of the acetyl groups under these conditions. Acetylated sporopollenin with a base hydroxyl value of 0.06 mmol/g after treatment with TFA consistently lost 0.04 mmol/g of acetyl groups. The loading of unprotected hydroxyls after treatment with TFA therefore was 0.10 mmol/g. This had to be accounted for when determining the loading of the hydroxy linker in later synthesis. Even though some of the acetyl groups were unstable to TFA treatment the loading of free hydroxyls was still very low. Previous investigations into the reactivity of these hydroxyls indicated that no

more than 38% of the naturally occurring hydroxyls could be acylated with Fmoc-protected amino acids (See Section 4.4.2). It was therefore assumed that the 2,4,5-trichlorophenyl ester of hydroxymethylphenoxyacetic acid would react to the same extent with such hydroxyls. This would result in only very small amounts actually being attached to the 0.04 mmol/g of free hydroxyls.

After Boc removal it was necessary to determine the amount of free amine available for further derivatisation. Fmoc analysis was carried out (Chapter 5, Section 5.2.8.3) in order to determine the loading of amine. After derivatisation with Fmoc-Cl, the support was reacted with ninhydrin (Chapter 5, Section 5.2.12) to ensure that all the free amine had reacted. Results of the Fmoc analysis revealed a loading of 0.22 mmol/g of free amine. However, elemental analysis showed that 0.45 mmol/g of amine was still attached to sporopollenin. (See Figure 4.13, The values in brackets refers to the loading of the functional groups in mmol/g determined by Fmoc analysis).

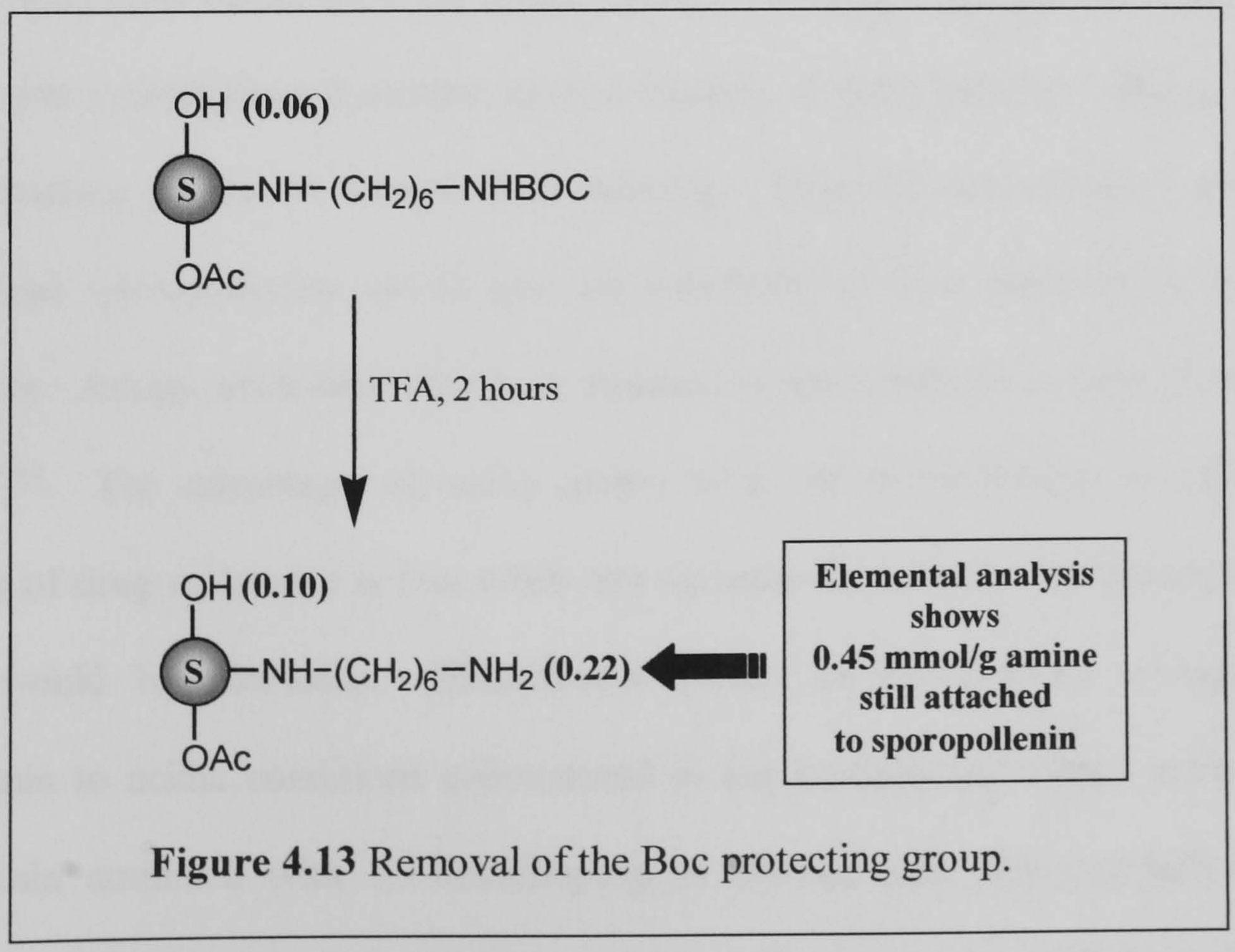


Figure 4.13 Removal of the Boc protecting group.

The reason for this difference in loading between the elemental analysis and Fmoc analysis could be due incomplete removal of the Boc protecting group or O to N migration of acetyl groups due to the close proximity between the hydroxyls and amine functionalites. Further investigations are necessary to investigate the reason for this low loading of free amine.

4.4.9 Functionalised Sporopollenin for use in Drug Delivery

Amination of sporopollenin with polyhydroxy amines would be a useful way of increasing the loading. High loadings of functional groups on solid supports are required for use in drug delivery to enable efficient attachment of drug molecules (Chapter 3, Section 3.5). Sporopollenin has been aminated with tris(hydroxymethyl)methylamine and sorbitolamine. Sporopollenin aminated with sorbitolamine gave a loading of between 1.53-1.73 mmol/g.[27d] If all the hydroxyls were derivatisable the potential loading of hydroxyls would be in the range 7.65-8.50 mmol/g. Sporopollenin aminated with tris(hydroxymethyl)methylamine gave a loading of 0.83 mmol/g. The potential hydroxyl loading would be around 2.49 mmol/g. Fmoc-Cl derivatisation of these functionalised sporopollenins would give an indication of how many hydroxyls are derivatisable. Amino acids have also been attached to sporopollenin in high yields (2-3 mmol/g).[27d,e] The advantage of using amino acids or sorbitolamine as points of attachment of drug molecules is that when they degrade in the body the decomposition products would be non-toxic. Preliminary studies of the stability of aminated sporopollenin to acidic conditions encountered in the stomach have been carried out. Sporopollenin aminated with 1,3-diaminopropane showed that after incubation with aqueous HCl (pH 1-3) at 37°C for 90 minutes, 92% of the amine remained attached.[27e]

Sporopollenin has also been aminated with tris-(2-aminoethyl)-amine polystyrene and its potential as a scavenger resin for electrophiles such as isocyanates is underway.[27d,e]

4.5 CONCLUSIONS

Sporopollenin can be prepared using different methods. Each of these methods have their advantages and limitations. The processing of sporopollenin used throughout the research was Method C (Section 4.1) due to the convenience of preparing sporopollenin with no impurities and ease of scale up. The sporopollenin prepared using this Method retained the shape of the original spores with no breakdown of their structure. Investigations into the swelling, filterability and stability characteristics of sporopollenin revealed that sporopollenin had physical properties which would make it an ideal support for solid phase organic synthesis. Sporopollenin exhibited only a small amount of swelling in the solvents tested, was easily filtered through grade 2-4 sinters, was shown to have a constant size and demonstrated greater mechanical stability than commercial PS-DVB resins. These properties would also make it an ideal support for drug delivery.

Functional group analysis gave an insight into the quantity of some of the functional groups present on the sporopollenin surface, namely acidic groups, hydroxyls unsaturated groups and ketones.

Sporopollenin was chlorinated, chloromethylated and aminated in order to find the most suitable attachment for applications in solid phase synthesis and drug delivery, To test the viability to solid phase synthesis it was initially planned to attach a hydroxybenzyl linker which would be used to synthesise 4-aminoimidazoles and purines.

Chlorination of sporopollenin with PCl_5 produced chlorinated sporopollenin with inconsistencies in loading. Chloromethylation of sporopollenin gave a consistent loading

which was comparable with the loadings of commercially available supports. Attempts to displace the chloride with the 4-hydroxybenzaldehyde linker under normal conditions used to displace chloride from chloromethylated PS resulted in only 26-37% displacement with the linker. It was suspected that chloromethylation of the double bonds present on the sporopollenin surface had resulted in the formation of aliphatic chlorides which were relatively unreactive.

Amination of sporopollenin resulted in loadings at useful and practical levels and the stability of the attachments was good. The exact nature of the linkage is still unknown but indications show that this linkage is non-basic and may be an amide. Sporopollenin was aminated with mono-*N*-Boc 1,6-hexanediamine in order to act as an attachment point for the hydroxybenzyl linker. Problems were encountered during Boc removal as the amount of derivatisable amine was low even though elemental analysis revealed that the loading of amine should be higher. This problem could be overcome by amination of acetylated sporopollenin with a hydroxy functionalised amine such as aminomethyl benzyl alcohol. This could then be used for direct attachment of the amino acid to be used in imidazole synthesis. Various cleavage conditions could then be tried to find the most suitable one.

This research has led to a better understanding of the surface reactivity of sporopollenin. Because the structure of sporopollenin is unknown it has been difficult to understand what is happening during the reaction of sporopollenin with the various reagents. This ground work is only the beginning and future work is necessary in order to develop a functionalised sporopollenin which could be used in solid phase organic synthesis, drug delivery and as a scavenger resin.

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5 EXPERIMENTAL SECTION

5.1 MATERIALS

Chemicals were purchased from the following companies, Sigma, Aldrich, Avocado, Fluka, BDH, Novabiochem, Fisher scientific and Advanced ChemTech. Anhydrous DMA and DMF were purchased from Aldrich in sure-sealed™ bottles for reactions involving sporopollenin and resins. When DMF was used with any substrate containing a fluorenylmethyl substituent, it was purged with nitrogen immediately before used to remove any dimethylamine (decomposition product) which may have been contaminating the solvent.[1] Dioxane, toluene and diethyl ether were dried over sodium wire and DCM was dried by treatment with $CaCl_2$ followed by distillation from calcium hydride.[2] Acetone was dried by distillation from anhydrous potassium carbonate.[2] Zinc Chloride was purified and dried by refluxing in dioxane with zinc dust, filtering hot and cooling to precipitate $ZnCl_2$. The precipitate was crystallised from dioxane and stored in a dessicator over P_2O_5.[2]

5.2 METHODS AND TECHNIQUES USED FOR ANALYSIS OF SPOROPOLLENIN DERIVATIVES

5.2.1 Elemental Analysis

Elemental analyses were performed on a Fisons instrument Carlo Erba EA 110 C H N analyser. Analyses were carried out in duplicate or triplicate for each sample and the values shown are the mean value of these analyses. Determinations were carried out by the analysis service, department of Chemistry, Hull University.

5.2.2 Infrared (IR) Spectroscopy

IR were recorded on a Perkin-Elmer Paragon 1000 fourier transform infra red spectrometer. Samples were intimately ground with anhydrous KBr (spectrosol grade) to produce discs. IR spectra were a result of 10 scans.

5.2.3 Ultraviolet-Visible (UV-VIS) Spectroscopy

UV absorption spectra were recorded on a lambda 10 ultraviolet-visible single beam spectrophotometer. One matched pair of quartz cells were used.

5.2.4 Microscopy

Microscope images were obtained using a Nikon Labaphot Upright Transmission Microscope. Scanning electron micrographs were obtained using a Leica Cambridge Stereoscan 360 Scanning Electron Microscope (SEM). The SEM images were performed by Tony Sinclair, Department of Chemistry.

5.2.5 Analysis of Chlorinated and Chloromethylated sporopollenin

5.2.5.1 Chloride determination using modified Volhards method [3,4]

METHOD A[3]

A sample of chlorinated sporopollenin (approximately 0.2 g), weighed accurately to 4 decimal places, was heated at 100°C with pyridine (3 cm^3) for 2 hours. The filtrate was collected quantitatively by recovering the sporopollenin by filtration and washing the sporopollenin with 50% v/v acetic acid/water mixture (2 x 15 cm^3). 6M HNO_3 (5 cm^3) was added to the combined filtrate and washings and the solution was stirred magnetically. Standard 0.1M $AgNO_3$ (5 cm^3) was added (standardised using NaCl)[4]

with stirring and the mixture was then left to stand for 5 minutes. Water (50 cm^3) was added followed by toluene so that one quarter of an inch of toluene was made on the surface. Iron (III) indicator (3 drops) was added, prepared by adding 124 g $NH_4Fe(SO_4)_2.H_2O$ to 100 cm^3 water. The mixture was then stirred whilst titrating with standard 0.1M NH_4SCN (standardised using standard 0.1M $AgNO_3$)[4] and the first tinge of red-brown indicated the end-point. The concentration of chloride released from the sporopollenin sample was calculated using Equation 1.

Number of moles of chloride in sample = Total number of moles of $AgNO_3$ added − Number of moles of $AgNO_3$ equivalent to number of moles of NH_4SCN required to reach end-point

Equation 1

The concentration of chlorine in sporopollenin was represented as mmol of chloride per gram of sporopollenin (mmol/g). Chloride analyses were carried out in triplicate for each derivatised sample.

METHOD B

The following modifications to the procedure were outlined in method A were carried out:

1 2M NaOH (5 cm^3) was used instead of pyridine for displacement of chloride from sporopollenin. Heating times of 2 and 8 hours were tried.

2 After the filtrate and washings were collected, the solution was decolourised with charcoal before the addition of $AgNO_3$.

3 After the addition of $AgNO_3$ solution, the mixture was protected from light and then heated with stirring for a few minutes to coagulate the AgCl. Any precipitate formed was removed by filtration and collected quantitatively. Water (50 cm^3) but no toluene was added before titration with NH_4SCN.

4 A less concentrated solution of NH_4SCN was used (0.02M instead of 0.1M).

5.2.5.2 Chloride determination using gravimetric analysis [4]

A sample of chlorinated/chloromethylated sporopollenin (0.2 g) was weighed accurately to 4 decimal places and heated to 100°C in 2M NaOH (5 cm^3) for 2 or 8 hours. The sporopollenin was collected by filtration and the filtrate was collected quantitatively. The sporopollenin was then washed with distilled water (3 x 30 cm^3). The combined filtrate and washings were acidified by the addition of 6M HNO_3 (5 cm^3) and then protected from light. 0.1M $AgNO_3$ (5 cm^3) was then added and the mixture was stirred with heating until coagulation of the precipitate had occurred. The cooled mixture was filtered through an oven dried, pre-weighed grade 2 sintered conical funnel and the collected AgCl precipitate was washed with a dilute nitric acid (3 cm^3). The precipitate was covered and dried in an oven at 120°C until a constant weight was achieved. This procedure was carried out in triplicate and the concentration of chloride was calculated as mmol/g.

5.2.6 Determination of the Hydroxyl Content of Sporopollenin and Derivatives

5.2.6.1 Fmoc Analysis

Derivatisation:[5a] Sporopollenin (0.05 g) was suspended in dry DCM (10 cm^3) and Fmoc-Cl was added and stirred for 5 minutes. Pyridine (same molar equivalent of Fmoc-

Cl used) was then added dropwise and stirred for 30 minutes.[5a] Sporopollenin was then collected by filtration and washed with DCM until no UV absorbing material was present in the filtrate. (This was detected by spotting the filtrate onto a TLC plate and visualising under UV) The sporopollenin was then dried in vacuo over P_2O_5 to a constant weight.

1 When the hydroxyl content was already known or indicated by other analytical methods, ie CHN analysis, then 2 equivalents of Fmoc-Cl and pyridine based on the loading of hydroxyls were used.

2 When the hydroxyl content was unknown an excess of Fmoc-Cl was used. For 0.05 g sporopollenin, 0.11 g (0.43 mmol) Fmoc-Cl was used and the same molar equivalent of pyridine was added.

Cleavage:[6] Piperidine (0.4 cm^3) and DCM (0.4 cm^3) were added to sporopollenin (approximately 5 mg weighed to 4 decimal places). The mixture was stirred and allowed to cleave for 1 hour and 30 minutes, at which point, MeOH (1.6 cm^3) was added. The sporopollenin was recovered by filtration and the filtrate was collected directly into a graduated flask. The sporopollenin was washed with DCM and the washings collected into the same graduated flask. DCM was then added to bring the final volume to 50 cm^3. The solution was then analyzed spectrophotometrically and the spectrophotometer was zeroed with a blank solution containing piperidine (0.4 cm^3), MeOH (1.6 cm^3) and DCM to bring the volume to 50 cm^3. The absorbance was measured at 301nm and the loading (number of hydroxyls) was given by Equation 2.

$$\text{Loading (mmol/g)} = \frac{A_{301} \times \text{Final Volume (ml)}}{7{,}800 \times \text{wt}}$$

Equation 2

Where: A_{301} = absorbance at 301nm

7800 = extinction coefficient of the piperidine-fluorenone adduct

wt = weight of sporopollenin in g.

Fmoc quantitation was carried out in duplicate or triplicate for each sample derivatised with Fmoc-Cl. Fmoc derivitisation was carried out in triplicate also.

5.2.7 Determination of the loading of amines on sporopollenin

5.2.7.1 Picric Acid Assay [7,8]

Aminated sporopollenin (0.02-0.03 g) weighed to 4 decimal places, was placed in a sintered filtration unit and soaked with DCM (10 cm^3). The sample was then treated with a saturated solution of picric acid in DCM (3 x 3 cm^3 x 2 minutes) and then washed with DCM until the filtrate was colourless. The sporopollenin was then treated with 0.1M DIEA (6 x 5 cm^3) which was collected directly into a graduated flask. The sporopollenin was then washed with ethanol and the volume made up to 250 cm^3 with ethanol. The spectrophotometer was zeroed with a blank containing DCM (6 cm^3) made up to 50 cm^3 with ethanol. The absorbance of the solution was then measured at 358 nm. The concentration of the diisopropylethylammonium-picrate was related to the loading of amine which was calculated using equation 3 derived from Beer-Lambert law.[4]

$$C = \frac{A_{358}}{\in . l}$$

Equation 3

Where C = mol dm^{-3}

A_{358} = Absorbance of solution at 358 nm

$\in$ = Molar absorption coefficient of diisopropylethylamine picrate

(14,500 $dm^3 mol^{-1} cm^{-1}$)

l = path length (1cm)

The loading of amine was expressed as mmol amine (free $-NH_2$) per gram of sporopollenin (mmol/g).

5.2.7.2 Elemental Analysis

Sporopollenin contains C, H and O and a little N. The loading of amine could be determined by nitrogen analysis. Elemental analyses were carried out in duplicate or triplicate. The loading was expressed as mmol amine per gram (mmol/g).

5.2.7.3 FMOC Analysis

The previous method of Fmoc derivatisation and cleavage was used (Section 5.2.6). The loading of the amine was determined by difference using equation 4.

Loading of amine (mmol/g)	=	Total concentration of FMOC (mmol/g)	−	Concentration of FMOC due to residual hydroxyls (mmol/g)

Equation 4

5.2.8 Determination of the Iodine Value of Sporopollenin [9]

Sporopollenin (approximately 0.2 g), weighed to 4 decimal places, was placed in a glass jar with a well fitted ground jointed stopper. Chloroform (10 cm^3) was used to transfer the sporopollenin sample into the jar. Wij's solution (20 cm^3) was added and the jar sealed by moistening the stopper with a small quantity of 10% KI solution. The jar was then allowed to stand in the dark at room temperature for the required reaction time.

After the appropriate time had elapsed, the sporopollenin was collected by filtration and the jar and the sporopollenin was washed with water (20 cm^3), 10% KI solution (15 cm^3) was added followed by water (60 cm^3) and then mixed by swirling. The free iodine was then titrated with standard 0.1N sodium thiosulphate solution[4] When the solution turned a pale-straw colour, 1% starch solution (1 cm^3) was added and the titration was continued until the end point was reached. (End point change was from blue to colourless.) A blank determination was also carried out. The titration's were either carried out in duplicate or triplicate. The iodine value was then calculated using Equation 5.

$$IV = \frac{12.69 \times N \times (B-T)}{W}$$

Equation 5

N = Exact normality of standard sodium thiosulphate solution

B = Volume of sodium thiosulphate solution required for the blank titration (cm^3)

T = Volume of sodium thiosulphate required in the sample titration (cm^3)

W = Weight of sample taken (g)

5.2.9 Determination of the acidity of sporopollenin[9]

Sporopollenin (0.2-0.3 g), weighed accurately to 4 decimal places, was added to a glass stoppered flask. Standard 0.5M NaOH solution[4] (25 cm^3) was added and stirred magnetically for the required reaction time. After the time had elapsed, the sporopollenin was collected by filtration and the flask was rinsed with distilled water (2 x 25 cm^3). The sporopollenin was then washed with further water (4 x 50 cm^3).

The filtrate was then titrated using standard 0.5M HCl solution[4] using screened methyl orange as the indicator. The titration was carried out as quickly as possible and the number of moles of NaOH consumed by sporopollenin was then calculated and expressed in mmol/g.

5.2.10 Tests for the Detection of Aldehydes and Ketones in Sporopollenin

5.2.10.1 Reaction of Sporopollenin with Brady's reagent[10]

Samples of sporopollenin (0.05 g) were refluxed with stirring in MeOH (1 cm^3) and Brady's reagent[10] (1 cm^3) for 30 minutes. The cooled sporopollenin was then collected by filtration and washed with water (4 x 20 cm^3), MeOH (4 x 20 cm^3) and then DMF until the filtrate was colourless. The sporopollenin was then washed with MeOH (4 x 20 cm^3), DCM (4 x 20 cm^3) and then dried in vacuo over P_2O_5 to a constant weight. Elemental analyses and IR spectra were recorded.

5.2.10.2 Reaction with Fehling's solution[11]

Two separate solutions for the test were prepared:

(1) Cu(II)SO_4 crystals (17.32 g) were dissolved in water which contained a few drops of dilute H_2SO_4. This was diluted to 250 cm^3.

(2) NaOH (12 g) and Rochelle salt (sodium potassium tartrate) (34.6 g) were dissolved in water and filtered through a sinter. The filtrate was then made up to 100 cm^3 with water.

Sporopollenin (0.05 g) and Fehling's solution 1 and 2 (1 cm^3 each) were heated on a boiling water bath for 5 minutes. The sporopollenin was then collected by filtration and a positive result had occurred if the solution turned yellow or red.

5.2.11 The Ninhydrin Colour Test for the Detection of Free Terminal Amino Groups on Derivatised Sporopollenin[6]

Stock solutions of the following were prepared,

(1) 500 mg ninhydrin in 10 cm^3 EtOH

(2) 40 g phenol in 10 cm^3 EtOH

(3) 1 cm^3 of 0.001M KCN solution diluted to 5 cm^3 with pyridine

A small sample of sporopollenin (20–30 mg) was placed in a test tube and 3 drops of each stock solution was added and the mixture was heated at 100 °C for 5 minutes. 60% aqueous EtOH (0.5 cm^3) was added and the mixture was shaken. This was then filtered through a Pasteur pipette containing a tight plug of cotton wool and collected into a small glass tube.

If the filtrate had a tinge of blue then there were free amino groups present. The colour of the solution was compared with a blank which was prepared by carrying out the above procedure using underivatised sporopollenin.

5.2.12 Determination of Phosphorus in Sporopollenin Derivatised with PCl_5 [12]

Stock solutions of the following were prepared,

(1) Ammonium molybdate (5 g/100 cm^3).

(2) Copper acetate buffer (0.125 g $CuSO_4.H_2O$ and 2.3 g sodium acetate added to 6 cm^3 of acetic acid and made up to 50 cm^3 with water).

(3) Reducing agent (p-methylaminophenol sulphate 5 g and 20 g sodium sulphite made up to 250 cm^3 with water).

A sample of chlorinated (PCl_5) was heated with 2M NaOH for 8 hours at 100°C. The sporopollenin was collected by filtration and the filtrated decolourised with charcoal. A sample of the solution (1 cm^3) was transferred to a test tube, copper acetete buffer (3 cm^3) and ammonium molybdate (1 cm^3) were added and shaken. If phosphorus was present then a yellow precipitate developed. After addition of the reducing agent (0.5 cm^3) an intense blue colour developed upon standing for 10 minutes.

5.3 PREPARATION OF SPOROPOLLENIN

METHOD A[7]

1 Lycopodium powder (250 g) were refluxed with mechanical stirring in acetone (750 cm^3) for 4 hours. The defatted spores were collected by filtration through a grade 4 sintered funnel and sucked dry. The spores were then refluxed with stirring with 6% aqueous KOH (850 cm^3) for 6 hours. The spores were then cooled to room temperature, recovered by filtration and washed with hot water. The treatment with fresh 6% aqueous KOH was then repeated. The spores were collected by filtration and then washed thoroughly with hot water (5 x 500 cm^3) and with hot ethanol (2 x 750 cm^3). The remaining material was then refluxed successively with ethanol (750 cm^3) and then dried in the air overnight.

The spores were then refluxed with stirring in 85% orthophosphoric acid (750 cm^3) for 7 days. The mixture was cooled to room temperature and diluted with purified water. The spores were collected by filtration and washed with water (5 x 750 cm^3), acetone (750 cm^3), 1M HCl (750 cm^3), 1M NaOH (750 cm^3), water (5 x 750 cm^3), acetone (750 cm^3) and finally ether (750 cm^3). The product was then shaken with 55% TFA in DCM (750 cm^3) for 24 hours after which it was filtered and washed with DCM, ethanol, water, ethanol and finally DCM.

The sporopollenin was then heated with stirring over 16 hours with a solution of ninhydrin, 80% aqueous phenol and a trace of KCN in ethanol-pyridine solution (750 cm^3). The cooled spores were then recovered by filtration and then washed with aqueous phenol, water, ethanol, acetic acid, ethanol and DCM. The final product was then dried in a vacuum oven at 80°C over P_2O_5 to a constant weight.

2 A sample of the sporopollenin (5 g) was taken and washed thoroughly with ethanol, water, ethanol and DCM, re-dried.

METHOD B

The method was the same as Method A except the steps which involved the use of phenol or acetic acid were missed out.

METHOD C

Lycopodium powder (250 g) was refluxed with mechanical stirring in acetone (750 cm^3) for 4 hours at 60°C. The cooled reaction mixture was then filtered and the spores were washed with acetone (4 x 100 cm^3) and sucked dry. The spores were then refluxed with mechanical stirring with 6% aqueous KOH (850 cm^3) for 6 hours at 100°C. The spores were cooled to room temperature, recovered by filtration and washed with hot water (4 x 250 cm^3), hot ethanol (2 x 250 cm^3) and hot water (4 x 250 cm^3). The treatment with KOH was repeated again with fresh 6% aqueous KOH solution. After this treatment the spores were collected by filtration and washed with hot water (8 x 250 cm^3) and hot ethanol (4 x 250 cm^3). The spores were then dried in the air and refluxed in ethanol (750 cm^3) for 2 hours and then after filtration refluxed in DCM (750 cm^3) for 2 hours after which time they were cooled, recovered by filtration and dried in the air. The spores were then stirred mechanically with 85% orthophosphoric acid (750 cm^3) for 5 days at 80°C. Upon cooling to room temperature, purified water was added and the spores were then collected by filtration and washed with cold water (4 x 250 cm^3), hot water (4 x 250 cm^3), hot ethanol (6 x 250 cm^3) and finally DCM (4 x 250 cm^3). The procedures and washing involving phosphoric acid treatment were repeated. The spores were then

sucked dry and then refluxed in a solution of ninhydrin (0.2 g) in ethanol (750 cm^3) for 2 hours, cooled, filtered and washed with water (4 x 250 cm^3), ethanol (4 x 250 cm^3) and DCM (2 x 250 cm^3).

Finally the spores were refluxed in DCM for 5 hours then collected by filtration and washed with further DCM (2 x 250 cm^3). The remaining material which was considered to be sporopollenin was dried *in vacuo* over P_2O_5 to a constant weight. This method of preparation of sporopollenin was repeated seven times.

METHOD D[13]

1 A sample of sporopollenin (20 g) prepared using Method C, Sample 06 was taken after the two phosphoric acid treatments and washings and dried in the air. The spores were then heated in 80% sulfuric acid (50 cm^3) at 80°C for 5 days. The cooled mixture was then transferred into water and the sporopollenin collected by filtration and washed with cold water (4 x 100 cm^3), hot water (4 x 100 cm^3), hot ethanol (4 x 200 cm^3) and finally DCM (4 x 250 cm^3).

2 Sporopollenin prepared using Method D.1 (0.5 g) was refluxed with 2M NaOH solution (20 cm^3) with stirring for 5 hours at 100°C. The cooled spores were recovered by filtration and then washed with hot water and hot ethanol until the filtrate was colourless. The product was dried *in vacuo* over P_2O_5 to a constant weight.

3 Sporopollenin prepared using Method D.2 (0.5 g) was refluxed with 2M KOH (20 cm^3) with stirring for 5 hours at 100°C. The cooled spores were then collected by filtration and washed with hot water and hot ethanol until the filtrate was colourless.

4 Large scale preparation of sporopollenin using Lycopodium powder (250 g) was used and the same procedure used in method B was carried out and then the extra step using 80% H_2SO_4 (750 cm^3) was added and the work up and washing procedures were the same as those used in Method D1 but scaled up.

METHOD E[14]

Lycopodium powder (0.5 g) were suspended in a mixture of 60% aq 4-MMNO and CHA (5 cm^3, 3:4 v/v) in a smooth glass mortar tissue grinder. After 15-20 minutes, 2.5 cm^3 of water was added and the suspension cycled past a teflon pestle with 10-15 up and down strokes. The total time in the reagent was 1-2 hours. The suspension was diluted 3-fold with water, centrifuged at 1500 x g for 5 minutes to pellet the grains and the supernatant fraction was discarded. Four additional washes with water, followed by centrifugation removed most of the MMNO. The washed pellet was then suspended in 6mM of MES buffer (12 cm^3) and then pelleted by centrifugation.

Any released fragments which floated to the surface were removed. The pellet was re-suspended in buffer (6 cm^3) and then cellulysin (60 mg), macerase (80 mg) and bovine serum albumin (8 g) were added to the suspension and incubated in a water bath with shaking for 2 hours at 27°C. Water (12 cm^3) was added to the solution followed by pelleting the particles by centrifugation. The pellet was then suspended in 0.15M NaCl and re-pelleted. The pellet was further suspended in 0.15M NaCl (10 cm^3), divided into 2 x 5ml portions and then added to a centrifuge tube which contained a 2 step gradient of 5 cm^3 each of 1.08 and 1.12 g / cm^3 of Percoll in 0.15M NaCl. This was then centrifuged at 3000 x g for 30 minutes. Non exine fragments were removed by washing the pellet with 0.15M NaCl 3 times. A small amount of water was added to the pellet which was

then loaded onto a 3 step gradient of CsCl (5 cm^3 each of 1.22, 1.39 and 1.45 g / cm^3 of CsCl). This was then centrifuged at 3000 x g for 30 minutes. The exine band was then recovered, collected by filtration and washed repeatedly with water to remove any gradient salts and then dried *in vacuo* over P_2O_5.

Elemental analyses and IR spectra were carried out on the sporopollenin prepared by the various methods. Elemental analyses were carried out in triplicate.

5.4 PHYSICAL STUDY OF SPOROPOLLENIN

5.4.1 Physical Appearance

Images of Lycopodium spores and sporopollenin prepared using Method C were obtained using scanning electron microscopy (SEM).

Lycopodium spores and sporopollenin prepared by Methods C and D were also viewed using a light microscope. The images were recorded using a digital camera.

5.4.2 Swelling Characteristics

Dried sporopollenin was mixed with various solvents and shaken for a few minutes. A drop of the mixture was taken and placed on a glass microscope slide and covered with a glass slip. The spore exines were then viewed using a light microscope, the image was recorded on a digital camera and the diameter of the spores were measured in μm The average size of the sporopollenin in each solvent was based on the measurements of 10 spores. The solvents used were water, DCM, methanol and DMF.

5.4.3 Filtering Experiments

Sporopollenin (2 g) was added to DMF (100 cm^3) and the mixture stirred magnetically for 2 minutes. The mixture was then filtered immediately through a grade 1 sintered funnel and the time taken for the solvent to pass through the sinter was recorded. The time taken for the solvent alone to pass through the sinter was also recorded. This procedure was then repeated using grades 2-4 sinters.

The above procedures were then repeated using the following solvents, DCM, THF, diethyl ether, methanol and water.

5.4.4 Drying Experiments

Drying experiments were carried out to examine the amount of water sporopollenin holds. Sporopollenin prepared by using one of the methods outlined and then dried in the air and then in a vacuum dessicator over P_2O_5 overnight. Samples of sporopollenin were weighed to 4 decimal places and then one of the following drying procedures were carried out. The weight of the sporopollenin sample was recorded after the drying processes and each one was carried out in triplicate.

1. Sporopollenin was dried in a vacuum oven at 80°C over P_2O_5 to a constant weight
2. Sporopollenin was dried in a microwave oven for 1 hour at 90°C.
3. Sporopollenin was dried in a vacuum dessicator over P_2O_5 to a constant weight

A thermogravimetric analysis of sporopollenin was also carried out. This was run at an increase of 30°C per minute to 900°C in a nitrogen atmosphere.

5.4.5 Mechanical Stability

Sporopollenin (0.05 g) was stirred magnetically in DCM (10 cm^3) at 250 1/min for 7 days using a magnetic stirrer. Samples were taken daily for microscope analysis. Wang resin (0.05 g) was also stirred magnetically for 1 day for microscope analysis.

5.5 REDUCTION OF SPOROPOLLENIN

5.5.1 Reduction with Lithium Aluminium Hydride[15]

$LiAlH_4$ pellets (3.6 g) were broken up and stirred in sodium dried dioxane (100 cm^3) under nitrogen. Sporopollenin was then added and the mixture was refluxed with stirring under nitrogen for 4 days. The mixture was cooled and then EtOAc (100 cm^3) was added slowly with cooling in ice / water mixture. Water (100 cm^3) was then slowly added followed by 2M H_2SO_4 (200 cm^3). The reduced sporopollenin was then recovered by filtration and then washed with water (3 x 100 cm^3), EtOH (3 x 100 cm^3) and DCM (3 x 100 cm^3). The sporopollenin was then dried to a constant weight *in vacuo* over P_2O_5.

5.5.2 Reduction with Sodium Borohydride[16]

Sporopollenin (1 g) was stirred in dry MeOH and then $NaBH_4$ (0.76 g) was added portionwise. When vigorous gas evolution had ceased, the reaction was stoppered and stirred for 48 hours after which the sporopollenin was collected by filtration and washed with MeOH (3 x 30 cm^3), MeOH / Water 1:1 (3 x 30 cm^3), water (3 x 30 cm^3), MeOH (3 x 30 cm^3) and finally DCM (3 x 30 cm^3). The sporopollenin was then dried to a constant weight *in vacuo* over P_2O_5.

IR spectra and Fmoc analysis (Section 5.2.7) were carried out on the reduced sporopollenins.

5.6 DERIVATISATION OF HYDROXYL GROUPS ON SPOROPOLLENIN

5.6.1 Chlorination of Sporopollenin

5.6.1.1 Chlorination with PCl_5[17]

PCl_5 (1.32 g, 6.34 mmol) was stirred to dissolution in dry DCM (35 cm^3) at 0°C. Anhydrous K_2CO_3 (0.88 g, 6.37 mmol) was then added followed by sporopollenin (1 g). The mixture was then stirred for 10 minutes at 0°C. The sporopollenin was then recovered by filtration and washed with DCM (4 x 20 cm^3) and water until no chloride was detected in the filtrate after addition of 0.1M $AgNO_3$ solution. The sporopollenin was then washed with MeOH (2 x 20 cm^3), DCM (4 x 20 cm^3) and then dried *in vacuo* over P_2O_5 to a constant weight. The loading of the chloride was then determined using chloride gravimetric and titrimetric analysis. (Section 5.2.6)

5.6.1.2 Chlorination with $SOCl_2$[18]

Sporopollenin (1 g) was stirred with diethyl ether (40 cm^3) at room temperature. Thionyl chloride (1.99 g, 16.7 mmol) was then added dropwise and then refluxed for 1 hour. The heat was then removed and stirred for a further 1 hour. The sporopollenin was recovered by filtration and washed with ether (4 x 20 cm^3) and then washes with water until no chloride was detected in the filtrate as AgCl after the addition of $AgNO_3$. The sporopollenin was then washed with MeOH (2 x 20 cm^3) and DCM (4 x 20 cm^3) before

drying *in vacuo* over P_2O_5 to a constant weight. The loading of chloride was determined using gravimetric and titrimetric analysis. (Section 5.2.6)

5.6.2 Attachment of 5-Chlorovaleric acid[19]

5-Chlorovaleric acid (0.82 g, 5.97 mmol) and PyBOP® (3.11 g, 5.97 mmol) were dissolved in anhydrous DMF. N-methyl morpholine (0.81 g, 7.96 mmol) was added and the mixture was stirred for 15 minutes at room temperature. Sporopollenin (0.5 g) was then added along with DMAP (0.05g, 0.40 mmol) and the mixture was stirred at room temperature for 24 hours. The sporopollenin was then collected by filtration and washed with DMF (4 x 20 cm^3), MeOH (4 x 20 cm^3) and finally DCM (4 x 20 cm^3). The final product was then dried *in vacuo* over P_2O_5 to a constant weight. The amount of 5-chlorovaleric attached was determined by gravimetric chloride analysis (Section 5.2.6).

5.6.3 Attachment of *N*-Fmoc Amino Acids to Sporopollenin

5.6.3.1 Attachment using Preformed Symmetrical Anhydride[5b]

Sporopollenin (0.5 g) was placed in a clean, dry flask and sufficient DMF was added to just cover the sporopollenin. In a separate flask Fmoc-amino acid (10 equivalents relative to the hydroxyl loading) was dissolved in dry DCM and a few drops of DMF was added to aid dissolution. DCC (5 equivalents relative to the hydroxyl loading) in dry DCM was added to the amino acid solution and the mixture was stirred for 20 minutes at 0°C with a $CaCl_2$ fitted to the flask. The DCM was then removed by evaporation and the residue was dissolved in DMF. This was added to the sporopollenin mixture. DMAP (0.1 equivalents relative to the hydroxyl loading) was then added and the mixture was stirred at room temperature for 24 hours. The sporopollenin was

recovered by filtration and washed with DMF (3 x 20 cm^3), DCM (3 x 20 cm^3), MeOH (3 x 20 cm^3) and DCM until the filtrate was washed free of Fmoc-amino acid. The sporopollenin was then dried *in vacuo* over P_2O_5 to a constant weight. The ninhydrin assay was performed to see if any free amino groups had been generated by cleavage of FMOC during the reaction. The loading of the amino acid was then determined by measuring the amount of Fmoc chromophore released upon treatment with piperidine. (Section 5.2.7)

5.6.3.2 Attachment using DCC / HOBt Method[1]

Sporopollenin (0.5g) was added to 9:1 (v/v) DCM / DMF (8 cm^3). In a separate flask the FMOC-amino acid, DCC and HOBt (3 equivalents each relative to the loading of hydroxyls on sporopollenin) were dissolved in the minimum amount of DMF. This was then added to the sporopollenin along with DMAP (0.1 equivalent relative to the loading of hydroxyls on sporopollenin) dissolved in the minimum amount of DMF. The flask was equipped with a drying tube and the mixture stirred for 24 hours at room temperature. The same washing and drying procedures and analysis were carried out as in 5.6.3.1.

5.6.3.3 The use of PyBOP® as an *in situ* Activating Agent[19]

The FMOC-amino acid and PyBOP® (3 equivalents each relative to the loading of hydroxyls on sporopollenin) were dissolved in the minimum amount of DMF. *N*-methylmorpholine (4 equivalents relative to the loading of hydroxyls on sporopollenin) was added and the mixture stirred for 15 minutes. Sporopollenin (0.5 g) was then added along with DMAP (0.2 equivalents relative to the hydroxyl loading) and the mixture

stirred at room temperature for 24 hours. The same washing, drying procedures and analysis were employed as in 5.6.3.1.

5.6.4 Acylation of Hydroxyls on Sporopollenin

5.6.4.1 Acylation using Acetic Anhydride

METHOD A[20]

Sporopollenin (1 g) and acetic anhydride (21.5 g, 0.25 mol) were refluxed with stirring for 4 hours. The cooled sporopollenin was then recovered by filtration and washed with ether (3 x 50 cm^3), water (3 x 50 cm^3), MeOH (3 x 50 cm^3) and acetone (3 x 50 cm^3). The product was then dried *in vacuo* over P_2O_5 until a constant weight.

METHOD B

Sporopollenin (0.01 g) was stirred in DCM (10 cm^3) and then acetic anhydride and TEA (1.5 equivalents relative to the loading of hydroxyls on sporopollenin) were added with stirring. DMAP (0.1 equivalents relative to the loading of hydroxyls on sporopollenin) was then added and the mixture stirred for 24 hours. The sporopollenin was then collected by filtration and washed with ether (3 x 20 cm^3), water (3 x 20 cm^3), MeOH (3 x 20 cm^3) and DCM (3 x 20 cm^3) and then dried over P_2O_5 to a constant weight.

METHOD C

This was the same as Method B but a longer reaction time of 48 hours was used.

METHOD D

This was the same as Method C but 2.25 equivalents of acetic anhydride and TEA were used.

5.6.4.2 Acylation using Acetyl Chloride

METHOD A

Sporopollenin (0.1g) was stirred in DCM (10 cm^3) and then acetyl chloride (3 equivalents relative to the loading of hydroxyls on sporopollenin) was added dropwise and stirred for 5 minutes. Pyridine (3 equivalents relative to the loading of hydroxyls on sporopollenin) was then added dropwise followed by the addition of DMAP (0.1 equivalents relative the loading of hydroxyls) and the mixture was stirred at room temperature for 48 hours. The sporopollenin was then recovered by filtration and washed with DCM (3 x 20 cm^3), MeOH (3 x 20 cm^3), water (3 x 20 cm^3), MeOH (3 x 20 cm^3), DCM (3 x 20 cm^3) and then dried *in vacuo* over P_2O_5 until a constant weight.

METHOD B

This was the same as Method A but 6 equivalents of acetyl chloride and pyridine were used. The reaction was carried out on a 0.1 g and 1 g scale.

METHOD C

Sporopollenin (0.1 g) was stirred with EtOAc (20 cm^3). Acetyl chloride and pyridine (6 equivalents each relative to the loading of hydroxyls on sporopollenin) and DMAP (0.1 equivalents) were added and the mixture was refluxed for 24 hours. The cooled

sporopollenin was then collected by filtration and the same washing procedures were used as in Method A.

The number of hydroxyls acylated using the above methods were determined using FMOC analysis.

5.6.5 Methylation of Sporopollenin

5.6.5.1 Methylation using Methyl Iodide[21]

Sporopollenin (0.1 g) was stirred with acetone (10 cm^3) and anhydrous K_2CO_3 (3 equivalents relative the loading of hydroxyls of sporopollenin) at room temperature for 1 hour. Methyl iodide (3 equivalents) was then added and the mixture was refluxed under nitrogen for 24 hours. The mixture was cooled and the sporopollenin was collected by filtration and washed with acetone (3 x 20 cm^3), water (3 x 20 cm^3), MeOH (3 x 20 cm^3) and DCM (3 x 20 cm^3). The final produce was then dried *in vacuo* over P_2O_5 until a constant weight.

5.6.5.2 Methylation using Dimethyl Sulfate[22]

METHOD A

Sporopollenin (0.1 g) was stirred with anhydrous K_2CO_3 (3 equivalents relative the loading of hydroxyls on sporopollenin) in acetone (10 cm^3) under nitrogen for 1 hour. Dimethyl sulfate (3 equivalents) were added and the mixture refluxed for 24 hours under nitrogen. The cooled spores were collected by filtration and washed and dried using the procedure outlined in 4.6.5.1.

METHOD B

This was the same as method A but 6 equivalents of K_2CO_3 and dimethyl sulfate was used.

The number of hydroxyls methylated using the above methods were determined using Fmoc analysis (see Secton 5.2.7).

5.7 CHLOROMETHYLATION OF SPOROPOLLENIN[23]

METHOD A

Sporopollenin (1 g) and dimethoxymethane (20 cm^3) was stirred at 35°C for 1 hour. The mixture was cooled to 0°C and $SOCl_2$ (2 g) was added dropwise followed by $ZnCl_2$ (0.5g) dissolved in dimethoxymethane (5 cm^3). The mixture was stirred at 35°C for 20 hours and the sporopollenin was then collected by filtration and washed with water until no chloride was detected in the filtrate. The sporopollenin was then washed with dioxane (3 x 50 cm^3), MeOH (3 x 50 cm^3), DCM (3 x 50 cm^3) and then dried *in vacuo* over P_2O_5 to a constant weight. This reaction was also carried out using 4 g of sporopollenin.

METHOD B

This was the same as method B but a larger amount of $ZnCl_2$ (2 g) was used.

The degree of chloromethylation using both methods was then determined by chloride gravimetric analysis (Section 5.2.6).

5.8 ATTACHMENT OF 4-HYDROXYBENZALDEHDYE TO CHLOROMETHYLATED SPOROPOLLENIN[24]

4-Hydroxybenzaldehyde and a base, either NaOMe, K_2CO_3 or NaH, (3 equivalents each relative to the loading of chloromethyl groups on sporopollenin) were stirred in an appropriate solvent (10 cm^3) at room temperature for 3 hours. Chloromethylated sporopollenin (0.18–0.20 g, weighed to 4 decimal places) was then added ad the mixture was stirred at 50°C for varying amounts of time.

After the appropriate time had elapsed the sporopollenin was collected by filtration and washed with water (3 x 20 cm^3) and the filtrate and aqueous washings were retained for chloride analysis. The sporopollenin product was then washed with EtOAc (3 x 30 cm^3), MeOH (3 x 30 cm^3), DCM (3 x 30 cm^3) and then dried in vacuo over P_2O_5 to a constant weight. The amount of chloride displaced under the reaction conditions was then determined by chloride gravimetric analysis of the filtrate (Section 5.2.6).

5.9 AMINATION OF SPOROPOLLENIN

5.9.1 Amination of Sporopollenin using 1,3-Diaminopropane[7]

METHOD A

Sporopollenin (3 g) was refluxed with stirring with 1,3-diaminopropane (10 cm^3) in toluene (25 cm^3) for 24 hours. The cooled sporopollenin was recovered by filtration and washed with toluene (3 x 50 cm^3), 2M HCl (2 x 50 cm^3), water (3 x 50 cm^3), MeOH (3 x 50 cm^3) and DCM (3 x 50 cm^3). The sporopollenin was then dried *in vacuo* over P_2O_5 to a constant weight. The loading of amine was determined by elemental analysis and by using the picric acid assay (Section 5.2.8).

METHOD B

Sporopollenin (0.1 g) was refluxed with 1,3-diaminopropane (3 equivalents relative to the loading of 1,3-diaminopropane found in method A) in toluene (20 cm^3) for 24 hours. The cooled sporopollenin was then collected by filtration and washed and dried using the same procedures as in method A. The loading of amine was determined by elemental analysis and by using the picric acid assay (Section 5.2.8).

5.9.2 Amination of Sporopollenin with n-Butylamine

METHOD A

Sporopollenin (0.1 g) and n-butylamine (3 equivalents based on the loading of 1,3-diaminopropane found in method A, 5.9.1) was refluxed in toluene (15 cm^3) for 24 hours. The cooled sporopollenin was collected by filtration and washed with DCM (3 x 30 cm^3), 2M HCl (3 x 20 cm^3), MeOH (3 x 20 cm^3) and DCM (3 x 20 cm^3) and then dried *in vacuo* over P_2O_5 to a constant weight. The loading of n-butylamine was determined by elemental analysis.

METHOD B

Sporopollenin (0.1 g) was stirred with DCC (3 equivalents relative to the amine loading found in section 5.9.1, Method A) in DCM (20 cm^3) for 20 minutes. n-Butylamine (3 equivalents) was added followed by DMAP (0.1 equivalents) and the mixture stirred for 24 hours at room temperature. The sporopollenin was collected by filtration then washed and dried using the same procedures outlined in Section 5.9.2, Method A. A blank, omitting DCC and DMAP was carried out. The loading of amine was determined by elemental analysis.

5.9.3 Amination of Sporopollenin with Mono-*N*-BOC-1,6-hexanediamine

Ion exchange resin (Cl form) was treated with excess 2M NaOH for 2 hours. The resin was then washed with water until free of chloride (tested by addition of $AgNO_3$) The resin was then washed with EtOH (2 x 30 cm^3), MeOH (2 x 30 cm^3) and ether (2 x 30 cm^3). Mono-*N*-Boc-1,6-hexanediamine hydrochloride (3 equivalents relative to the loading of 1,3-diaminopropane found in section 5.9.1, Method A) was dissolved in methanol and the ion exchange resin was then mixed with the amine until addition of $AgNO_3$ showed that all the chloride had disappeared. The resin was separated form the solution by filtration and then washed with MeOH (2 x 30 cm^3). The MeOH was then removed by evaporation and the amine was then co-evaporated with MeOH a further 3 times. Toluene was then added to the resulting residue followed by sporopollenin (0.1 g) and the mixture was refluxed with stirring for 24 hours. The sporopollenin was then collected by filtration and washed and dried using the method outlined in section 4.9.2. The loading of amine was determined by elemental analysis. The reaction was also carrie out on a larger scale using 0.7 g and 1 g of sporopollenin.

5.9.4 Stability Studies of the Aminated Sporopollenin

Sporopollenin aminated using the methods outlined in this section were exposed to various acidic and basic conditions.

5.9.4.1 TFA

Sporopollenin (0.05 g) was stirred with varying concentrations of TFA (2 cm^3) for 24 to 48 hours. TFA and TFA / DCM (1:1) was used.

5.9.4.2 Sodium Hydroxide

Sporopollenin (0.05 g) was stirred with 2M NaOH solution (5 cm^3) at room temperature for 4 days or refluxing temperature for 24 hours.

5.9.4.3 Hydrochloric acid

Sporopollenin (0.05 g) was stirred with 2M HCl (5 cm^3) at room temperature for 4 days or refluxing temperature for 24 hours.

The amount of amine still attached after these treatments was determined using elemental analysis.

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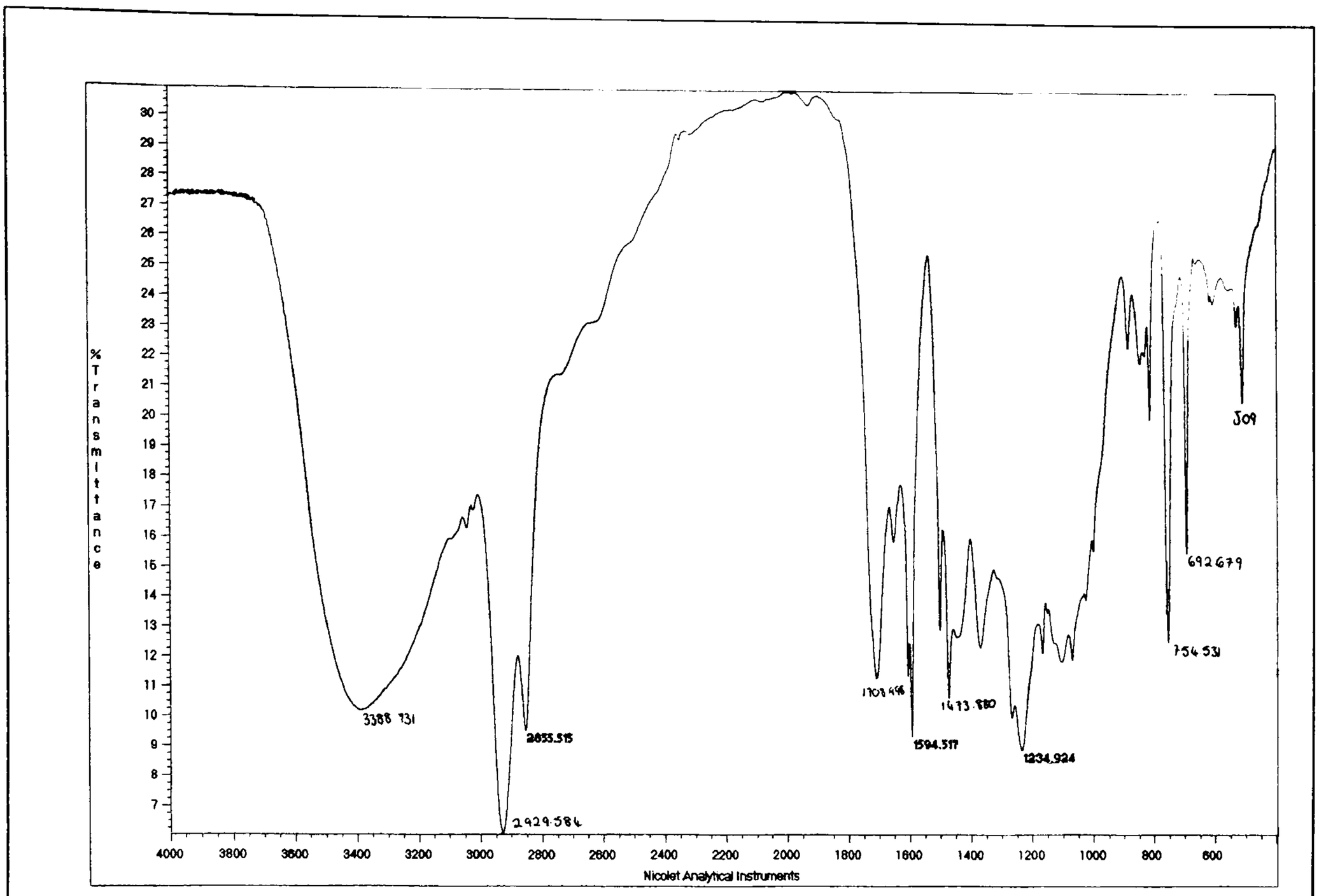
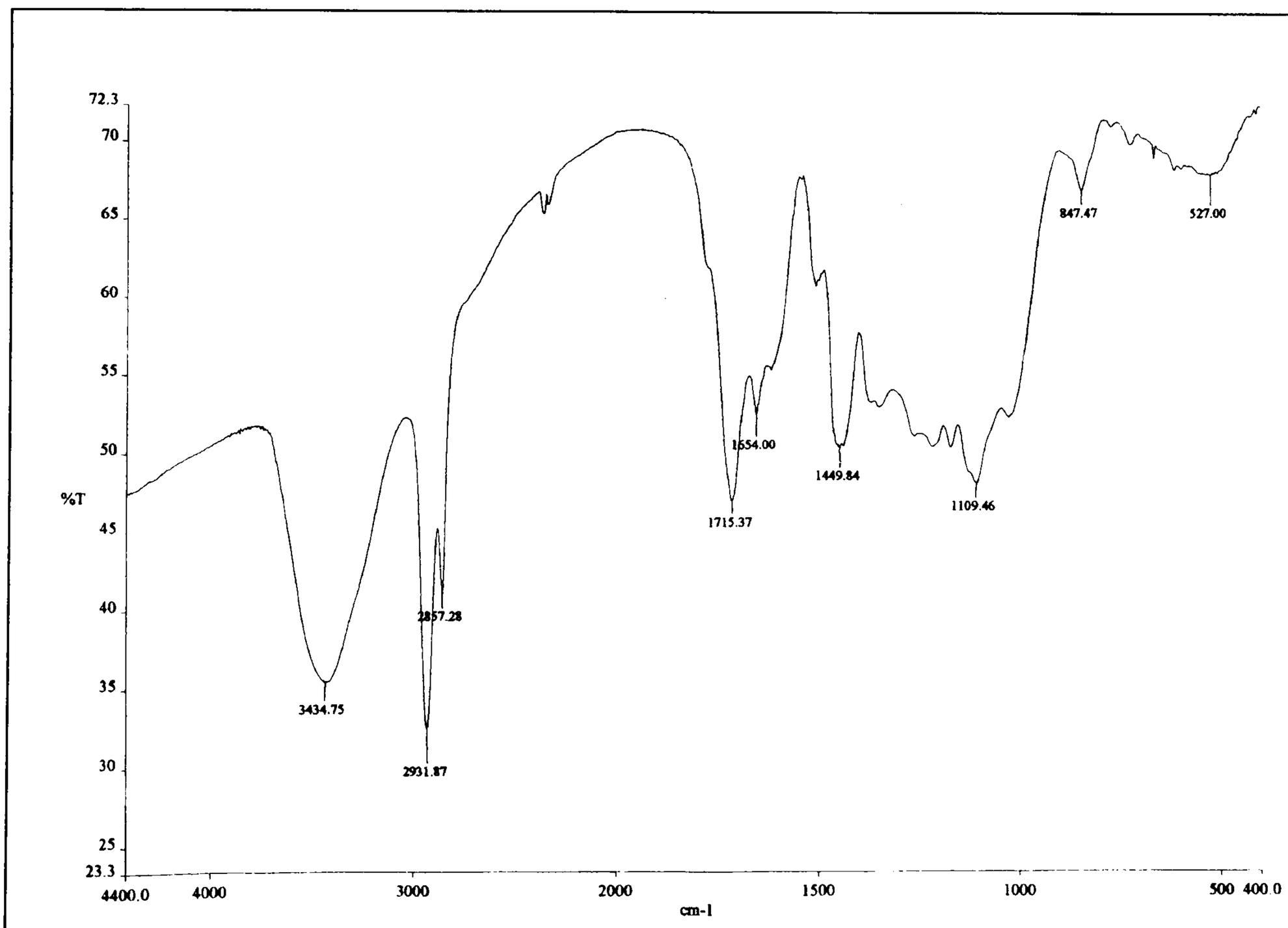
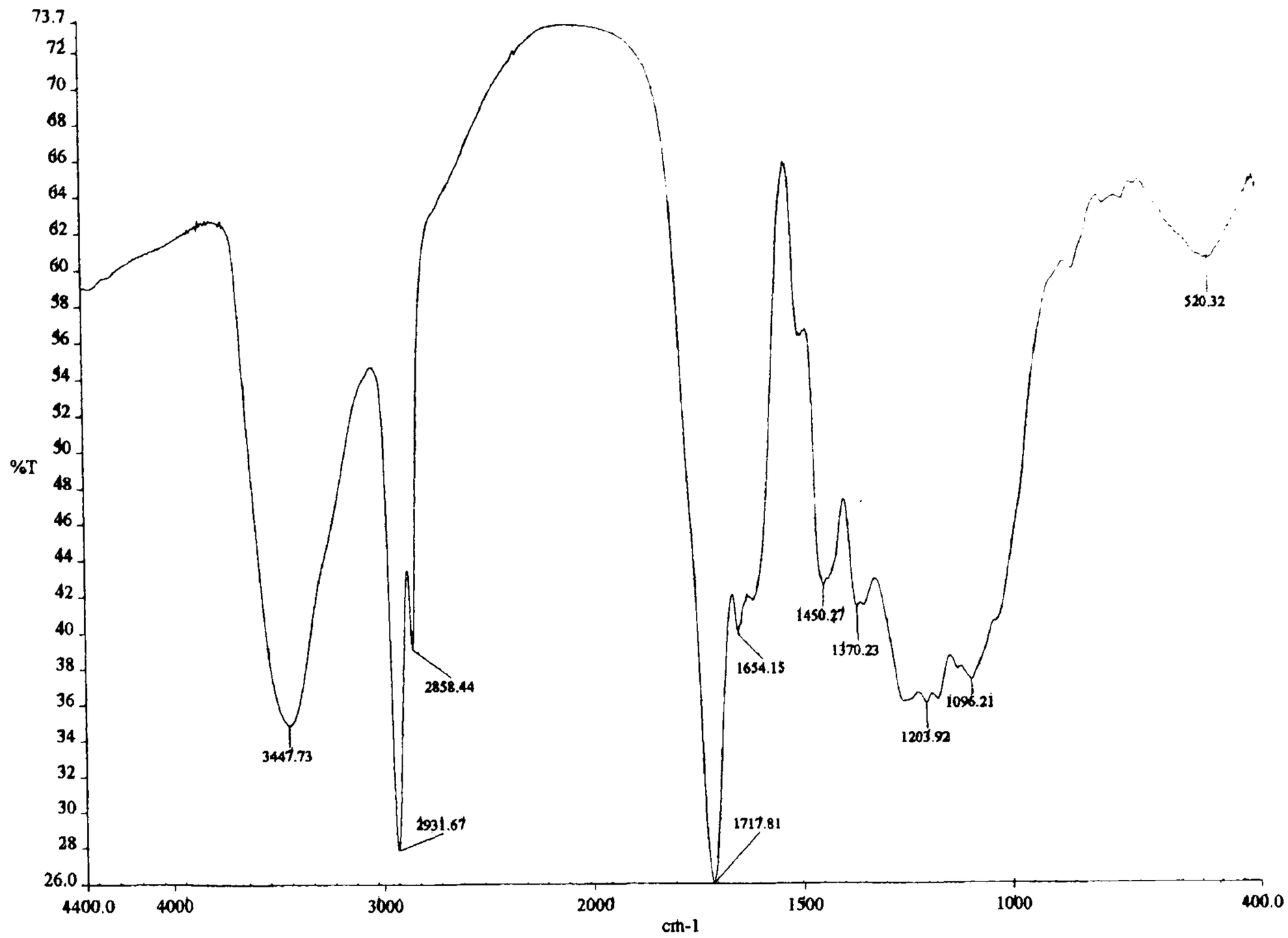


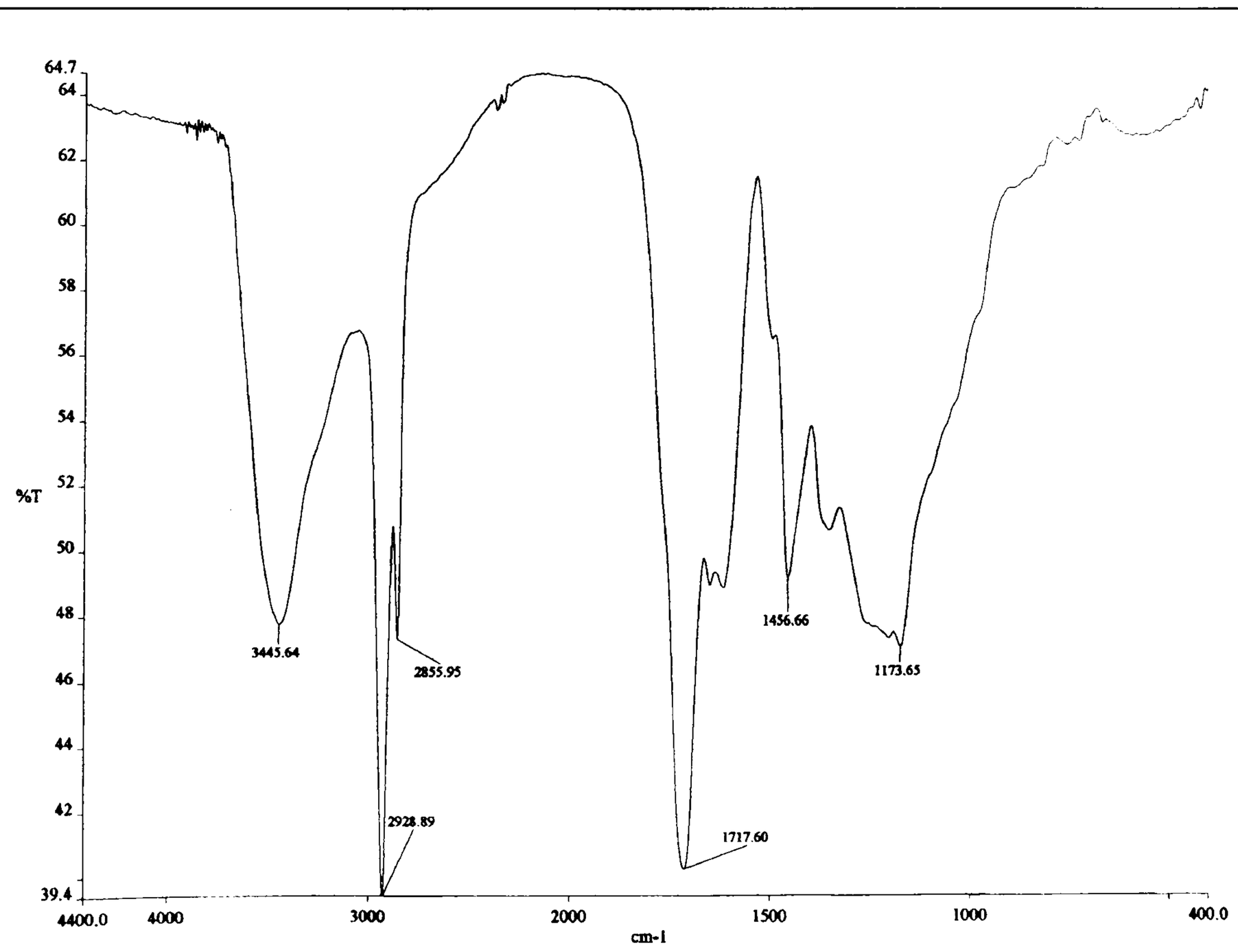
IR 1 Sporopollenin (Sample 01, Method A).



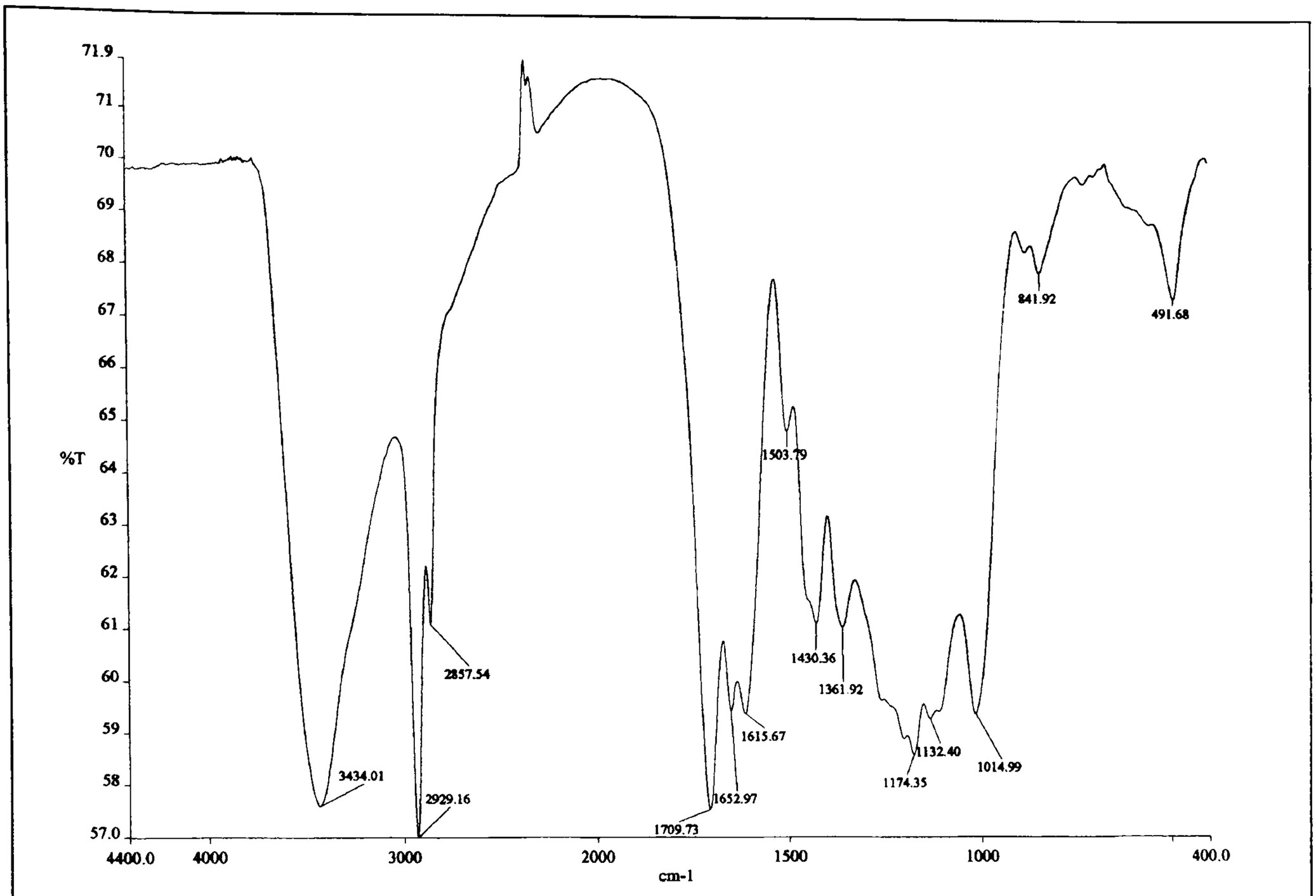
IR 2 Sporopollenin (Sample 01. Method A after re-wash).



IR 3 Sporopollenin (Sample 02, Method B).



IR 4 Sporopollenin (Sample 03, Method C).



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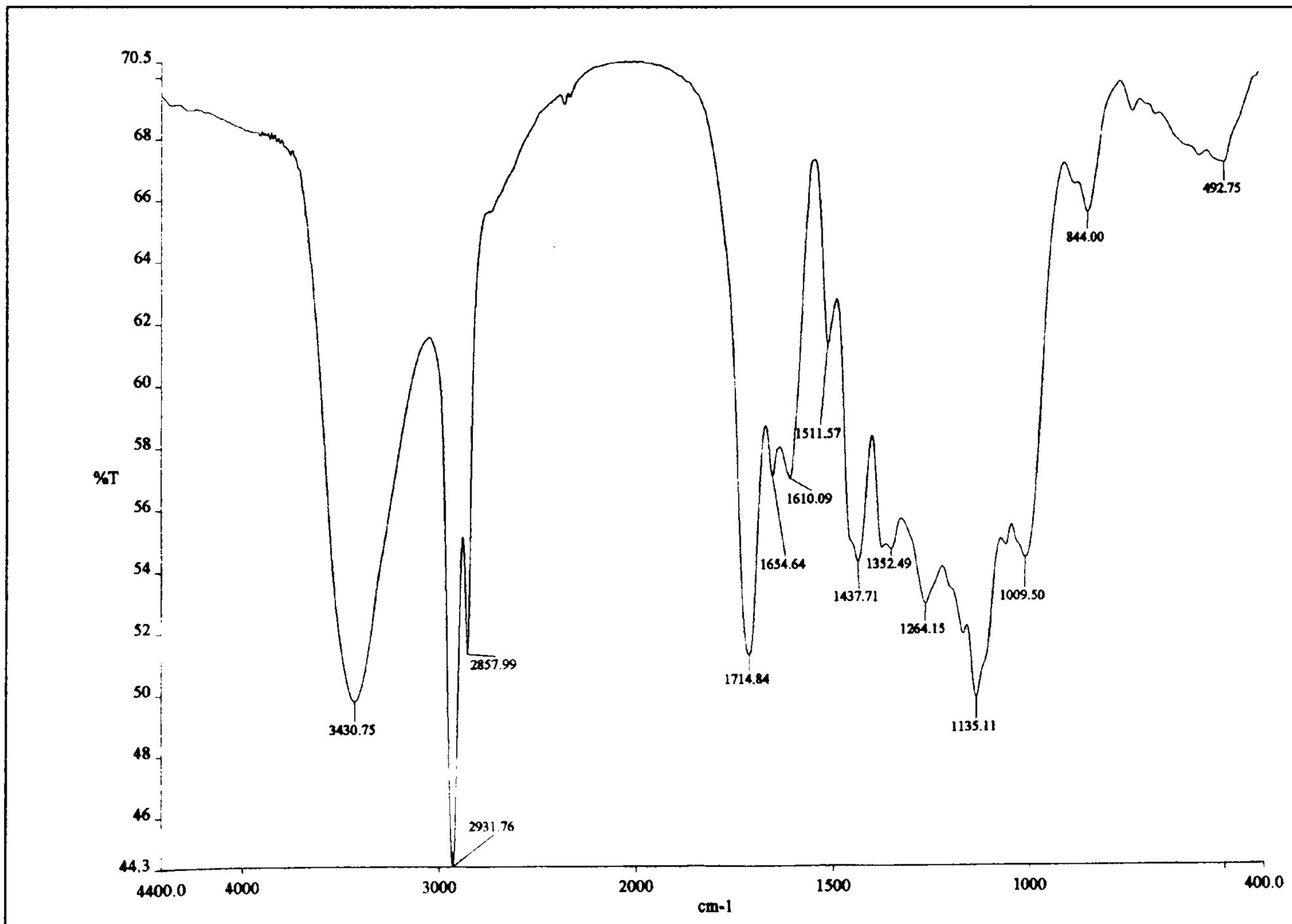

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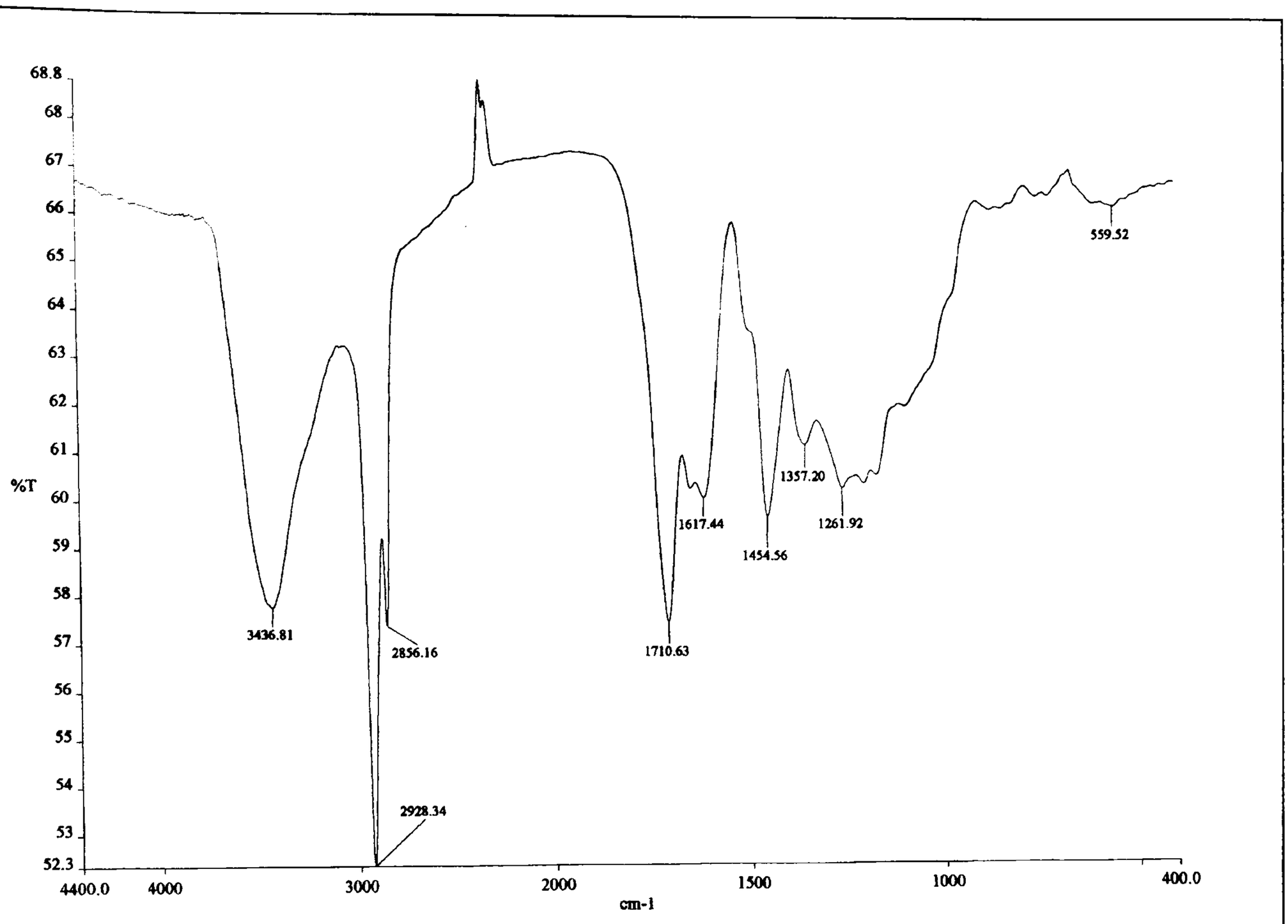
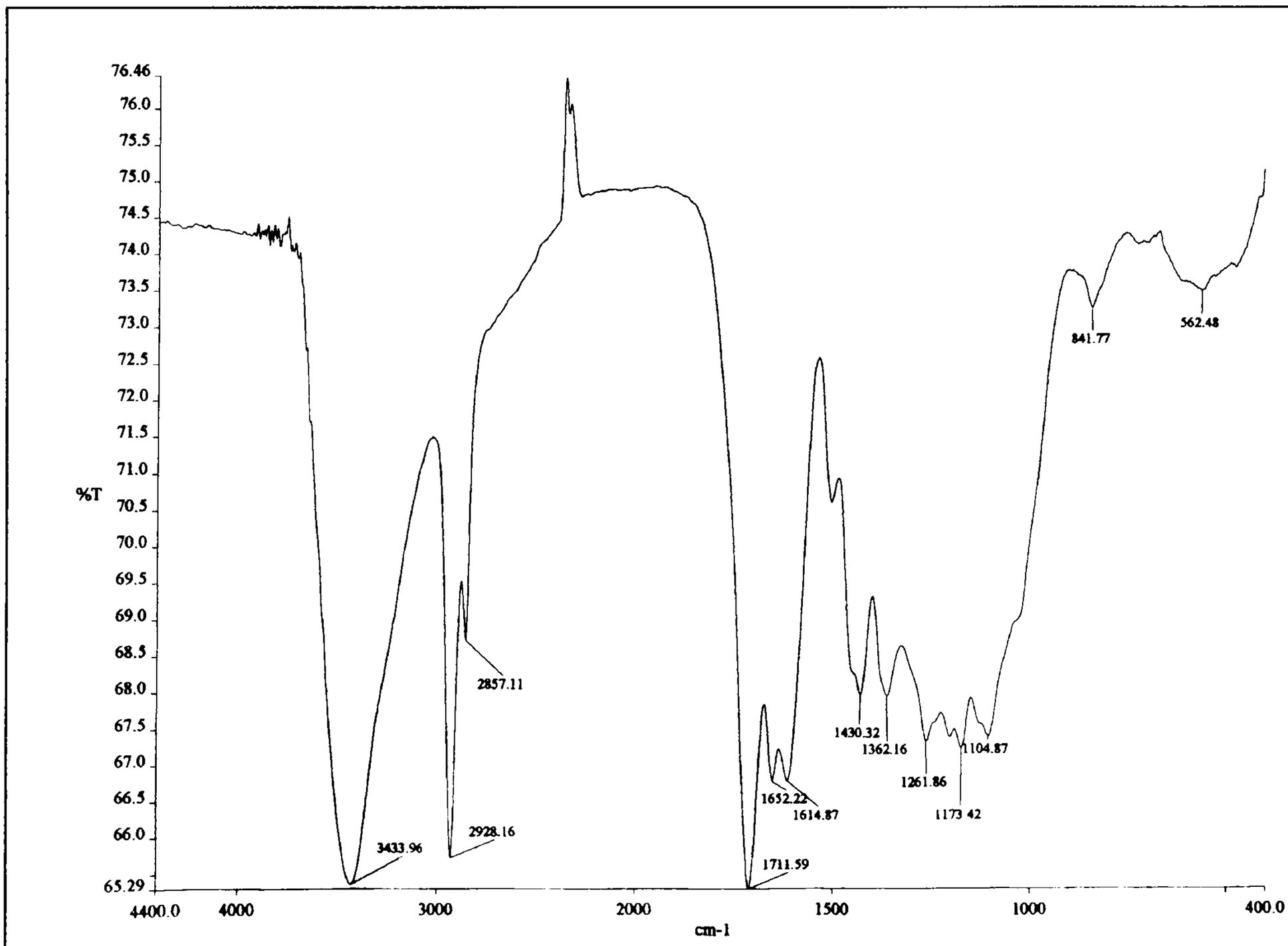
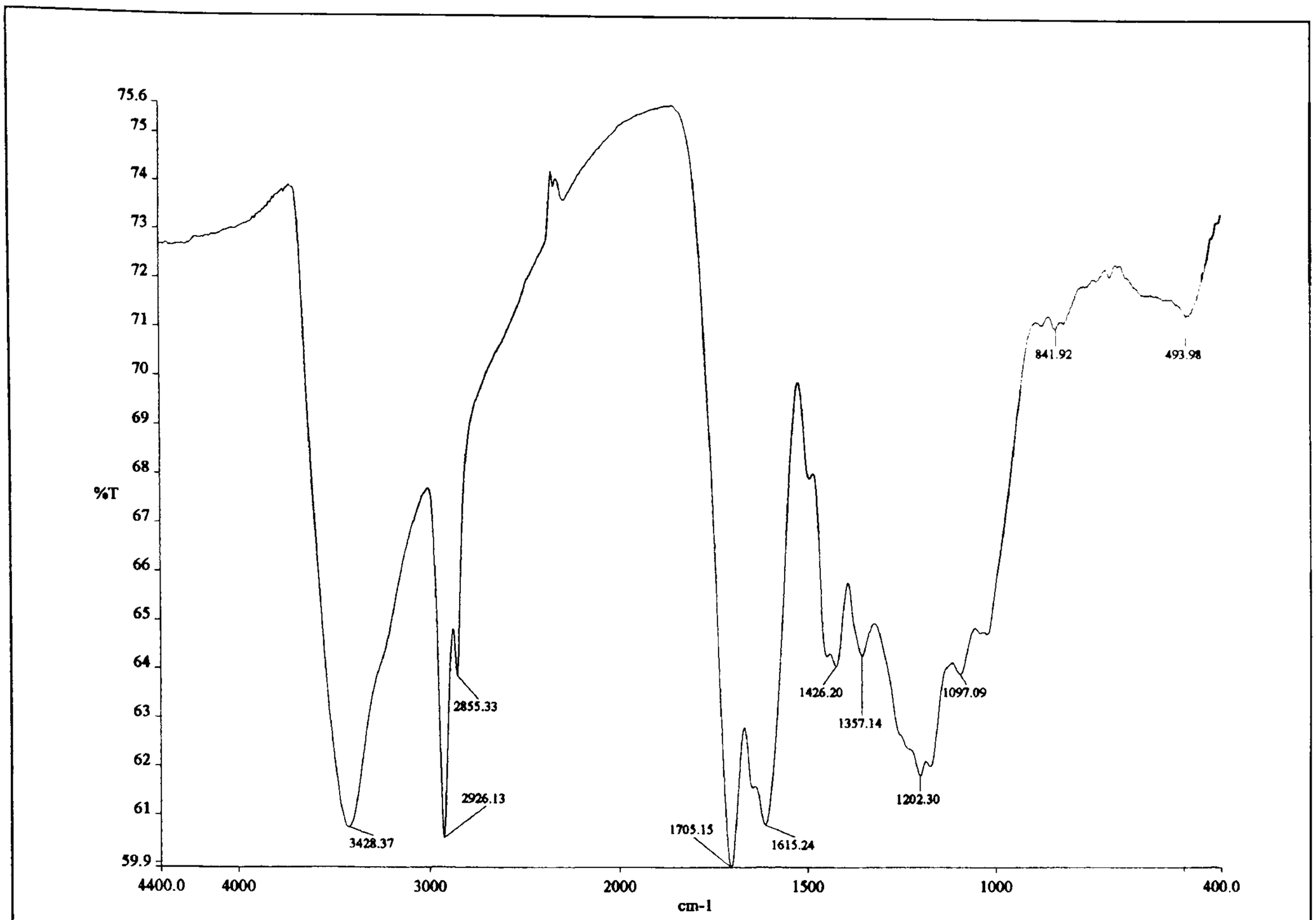


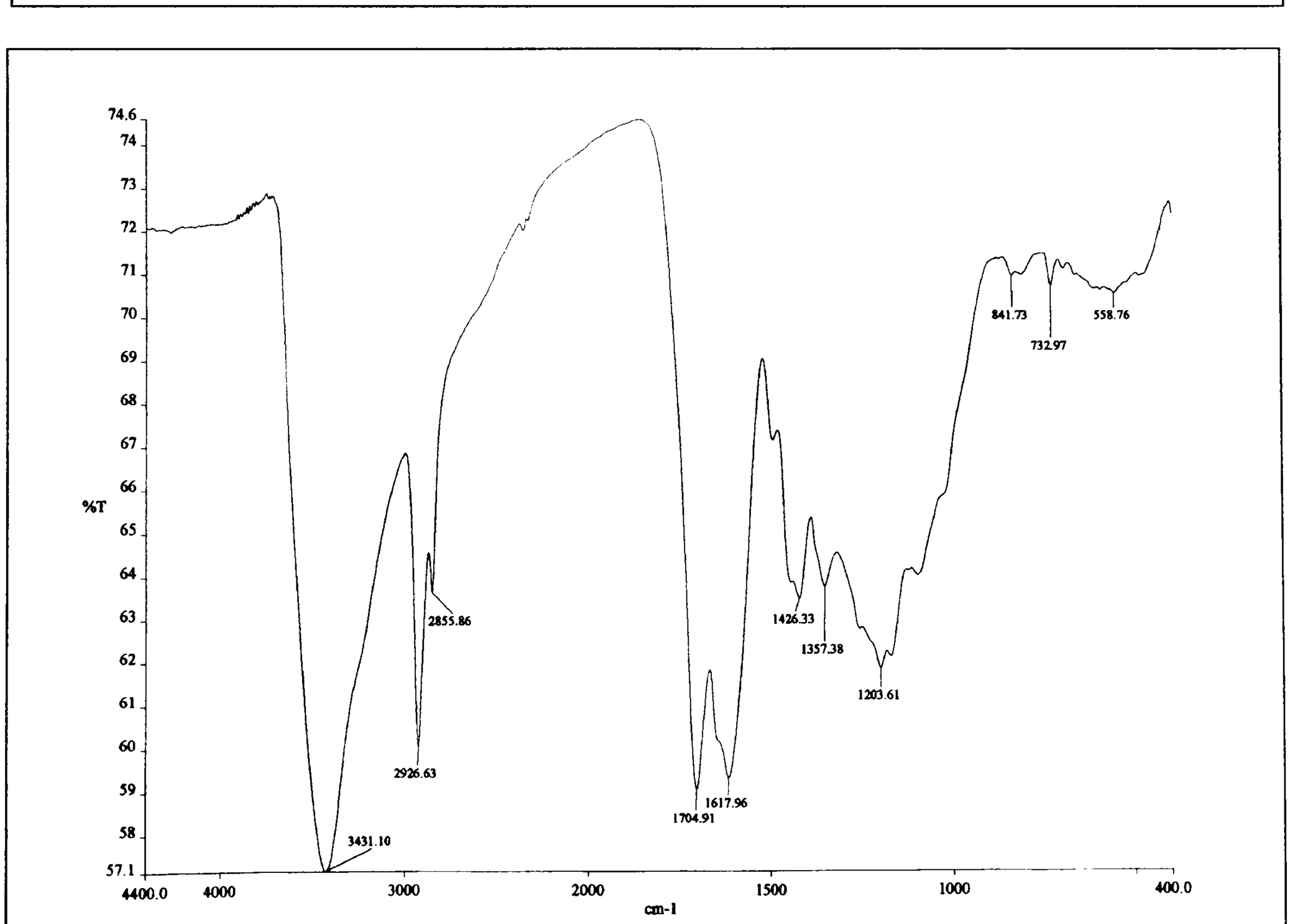
IR 7 Sporopollenin (Sample 06, Method C).



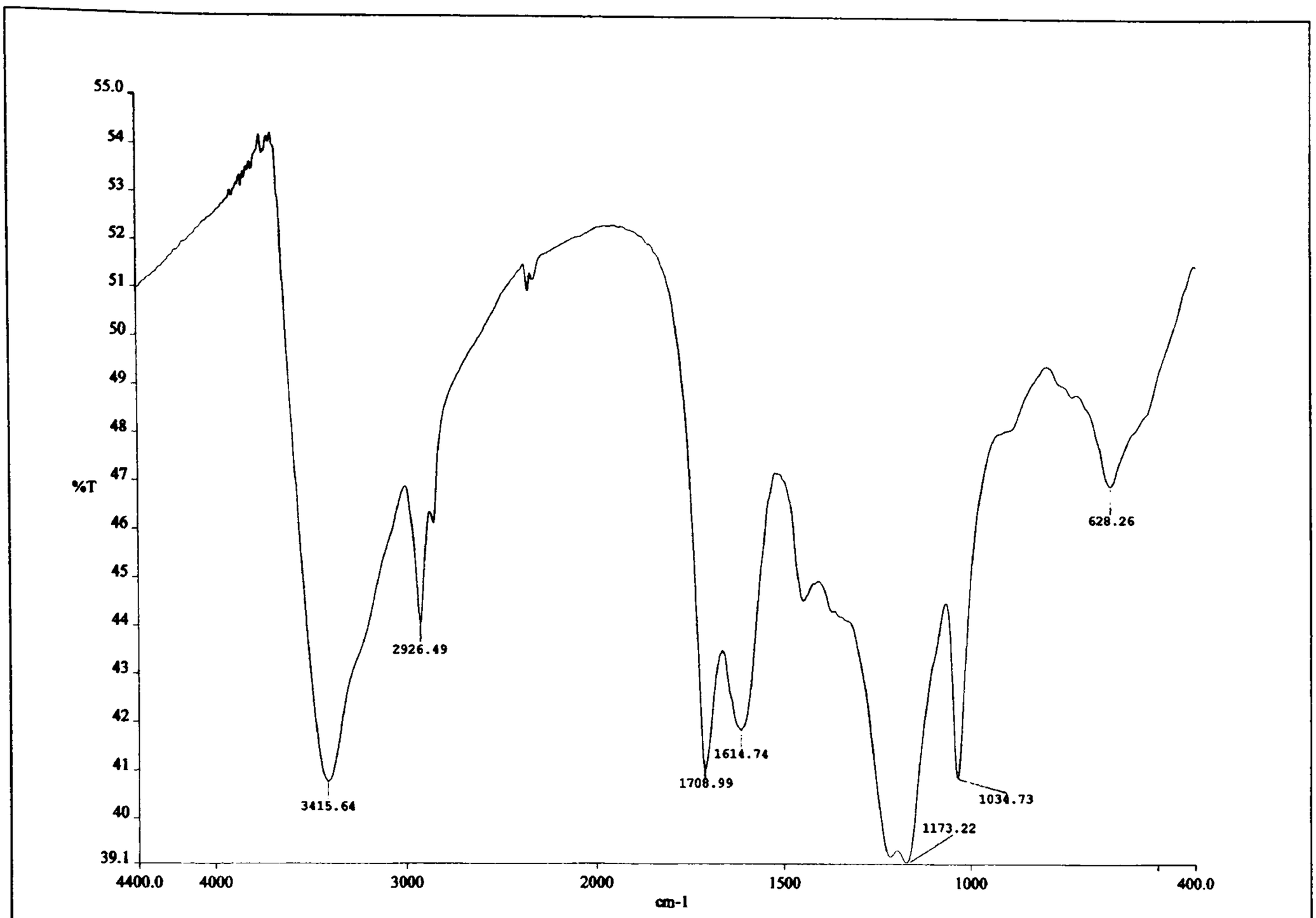
IR 8 Sporopollenin (Sample 07, Method C).



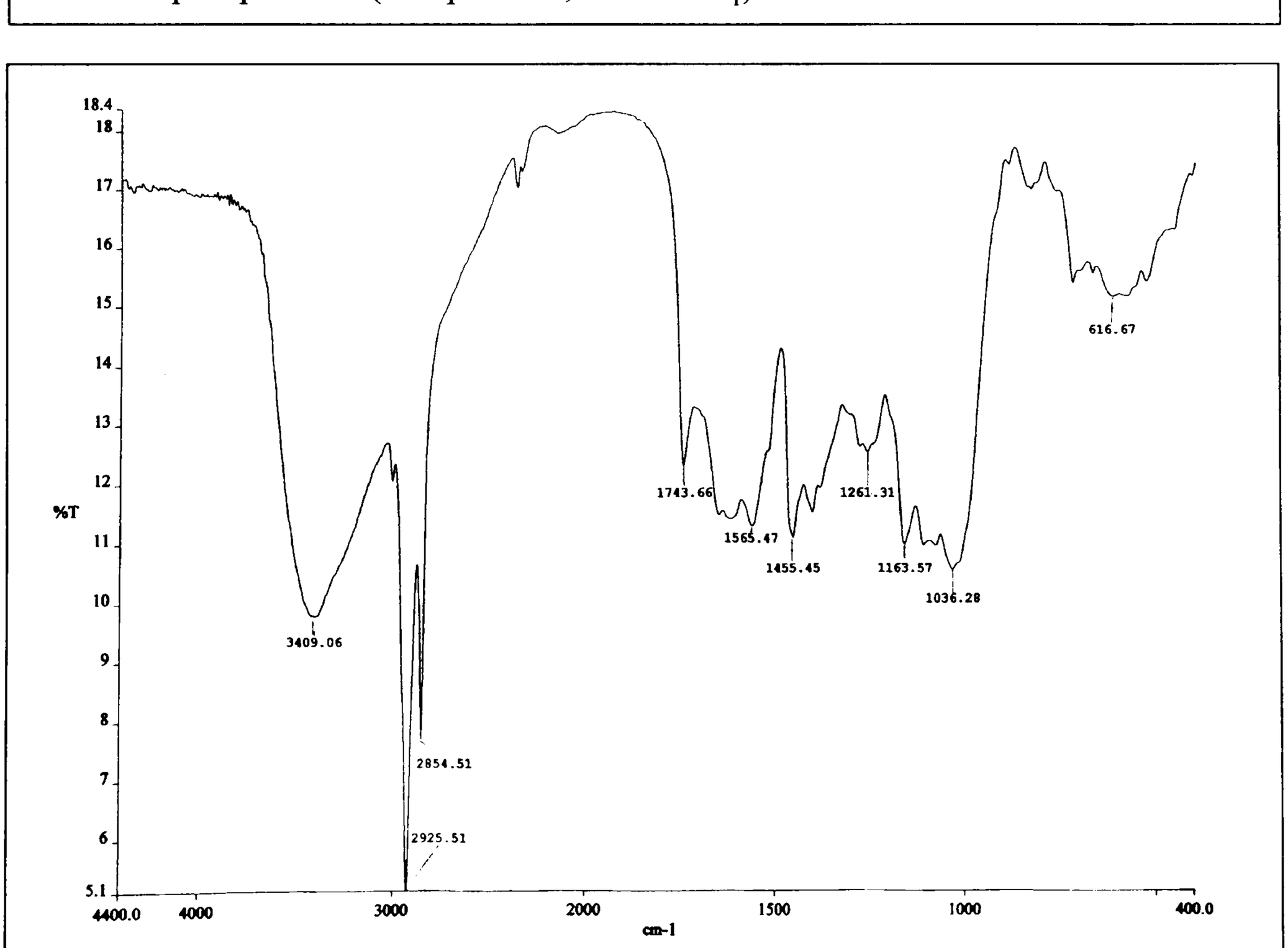
IR 9 Sporopollenin (Sample 08, Method C).



IR 10 Sporopollenin (Sample 09, Method C).



IR 11 Sporopollenin (Sample 10.1, Method D_1).



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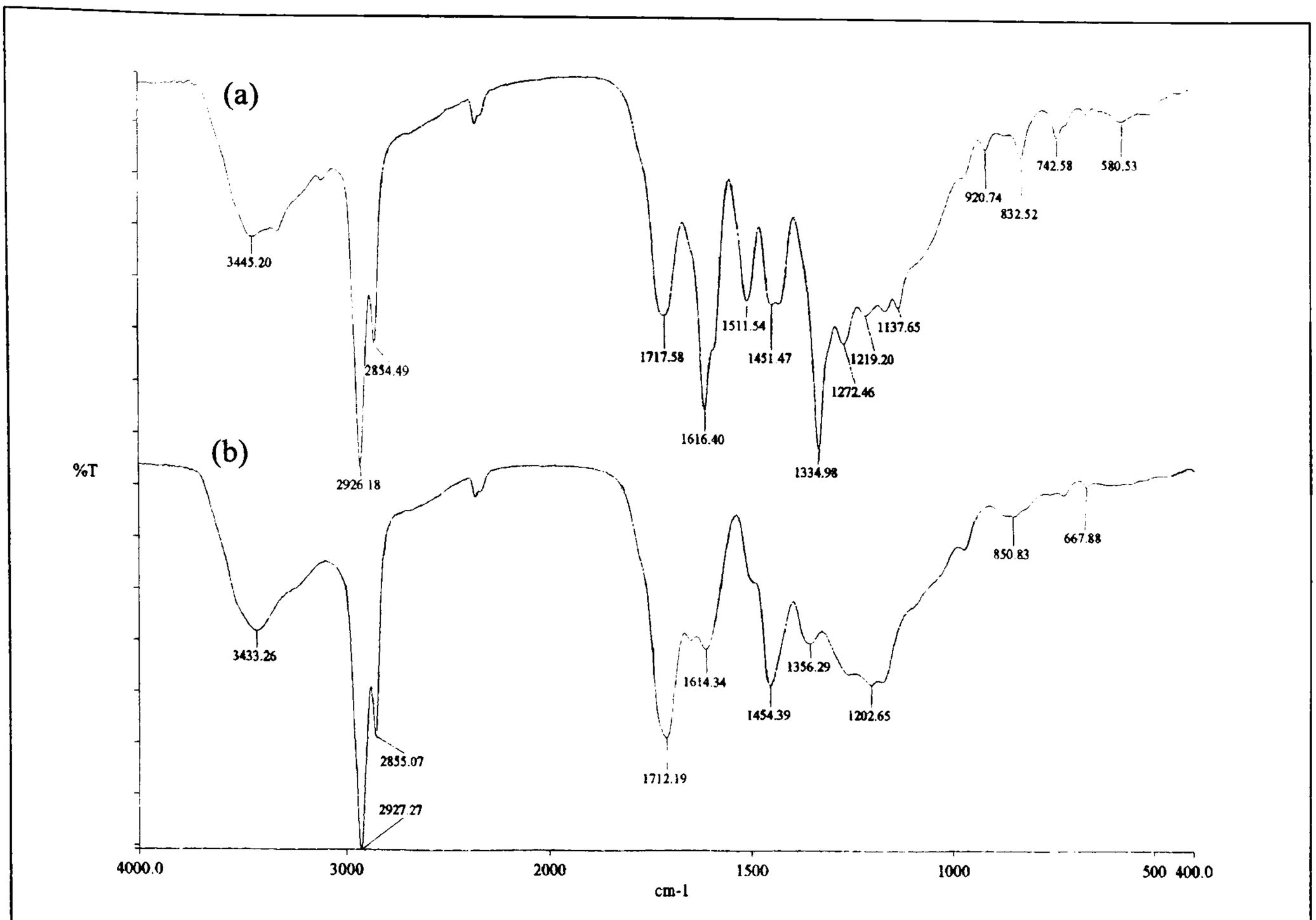
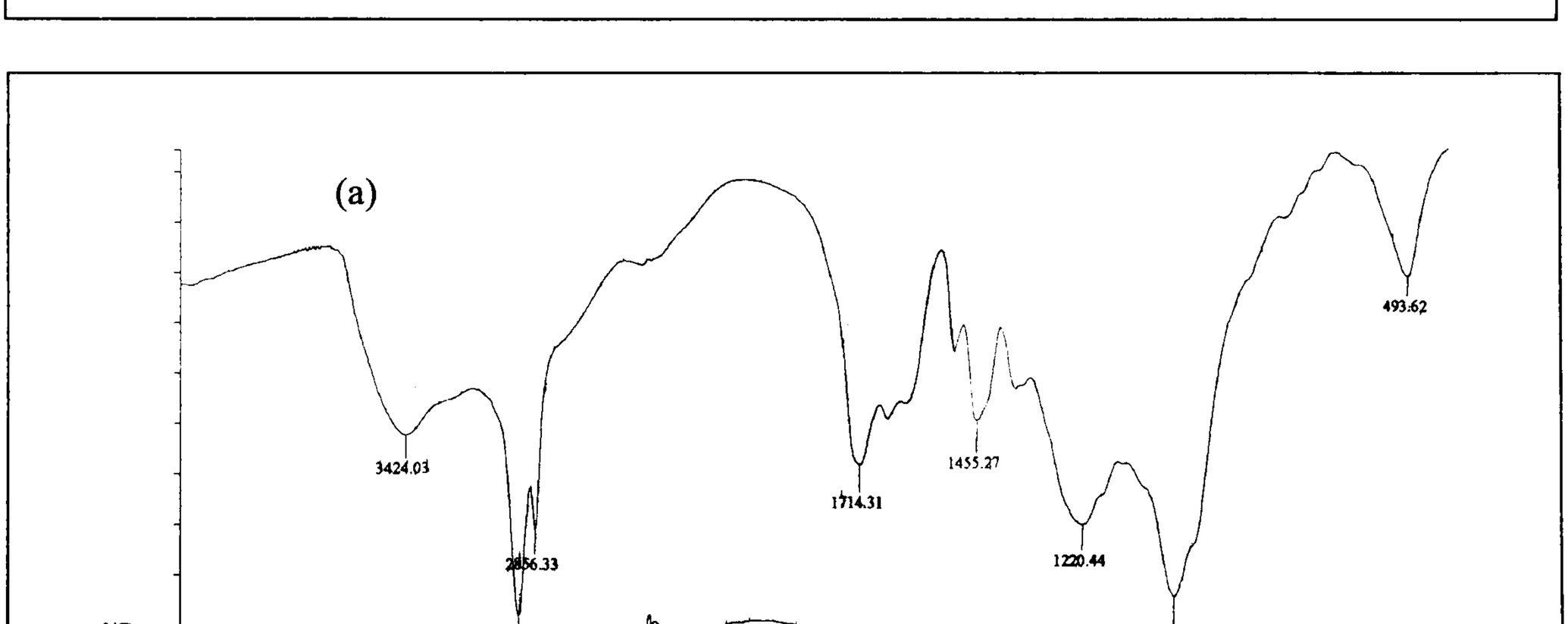
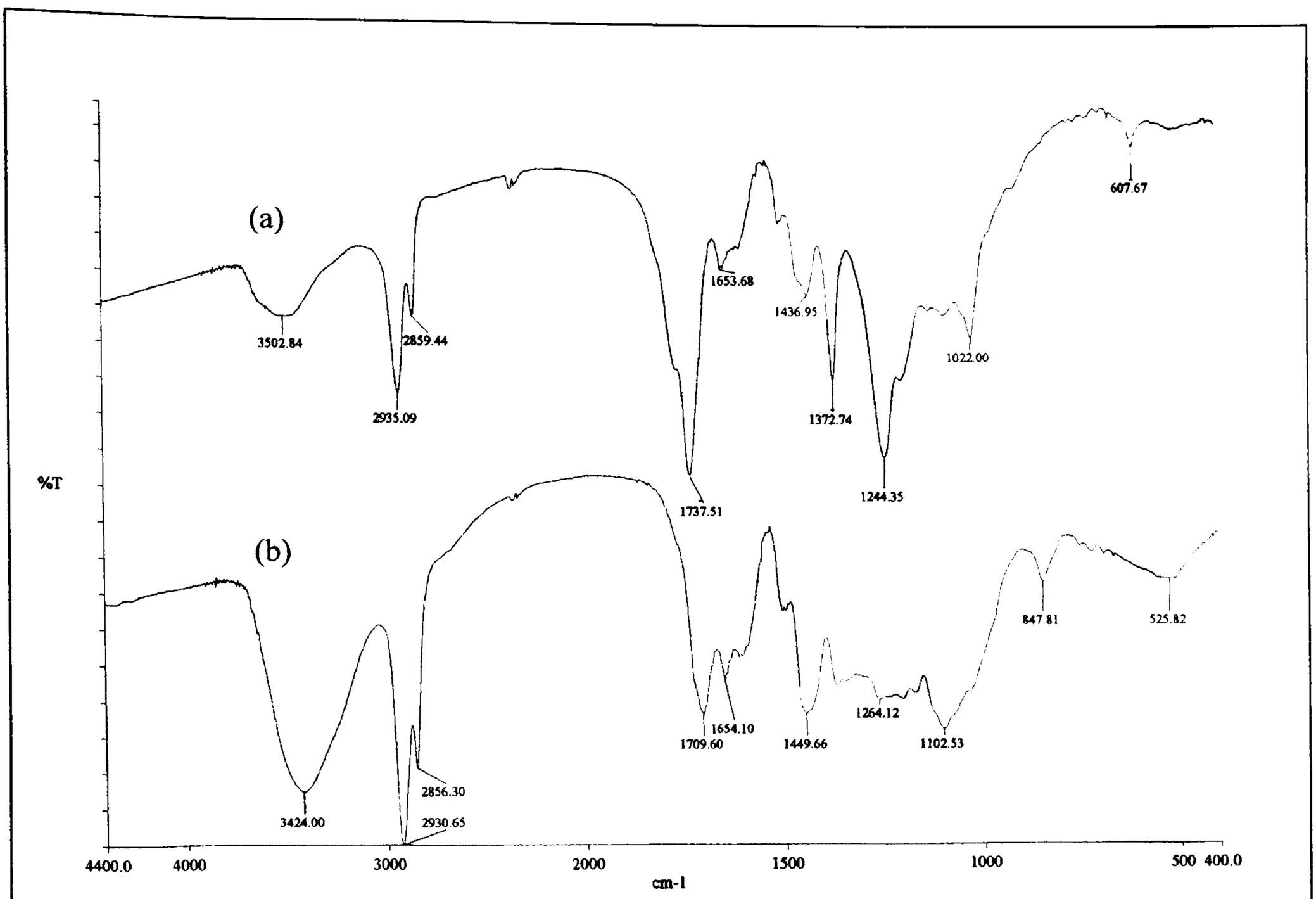


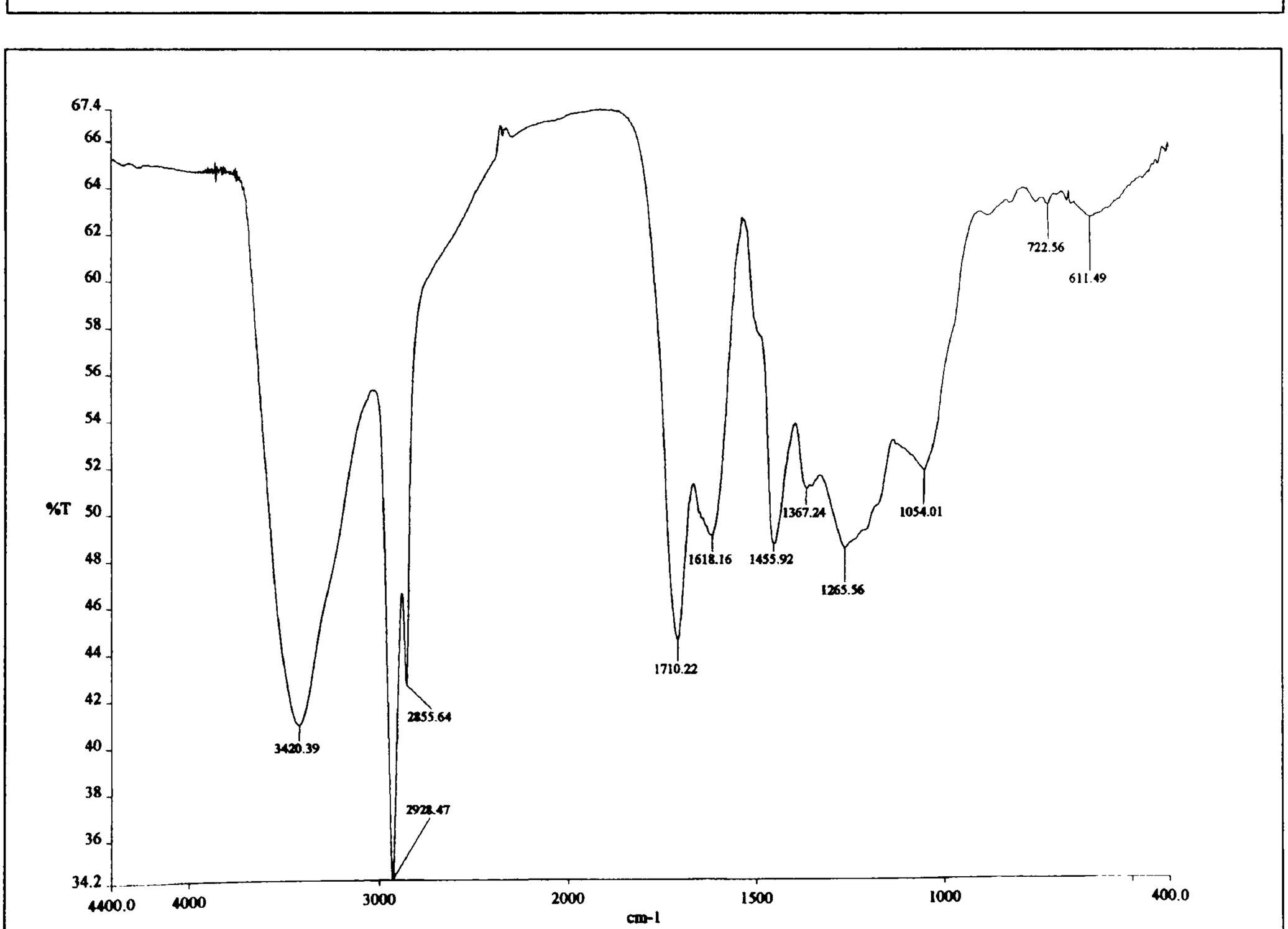
IR 13 (a) Sporopollenin derivatised with 2,4-DNPH, (b) Underivatised Sporopollenin.



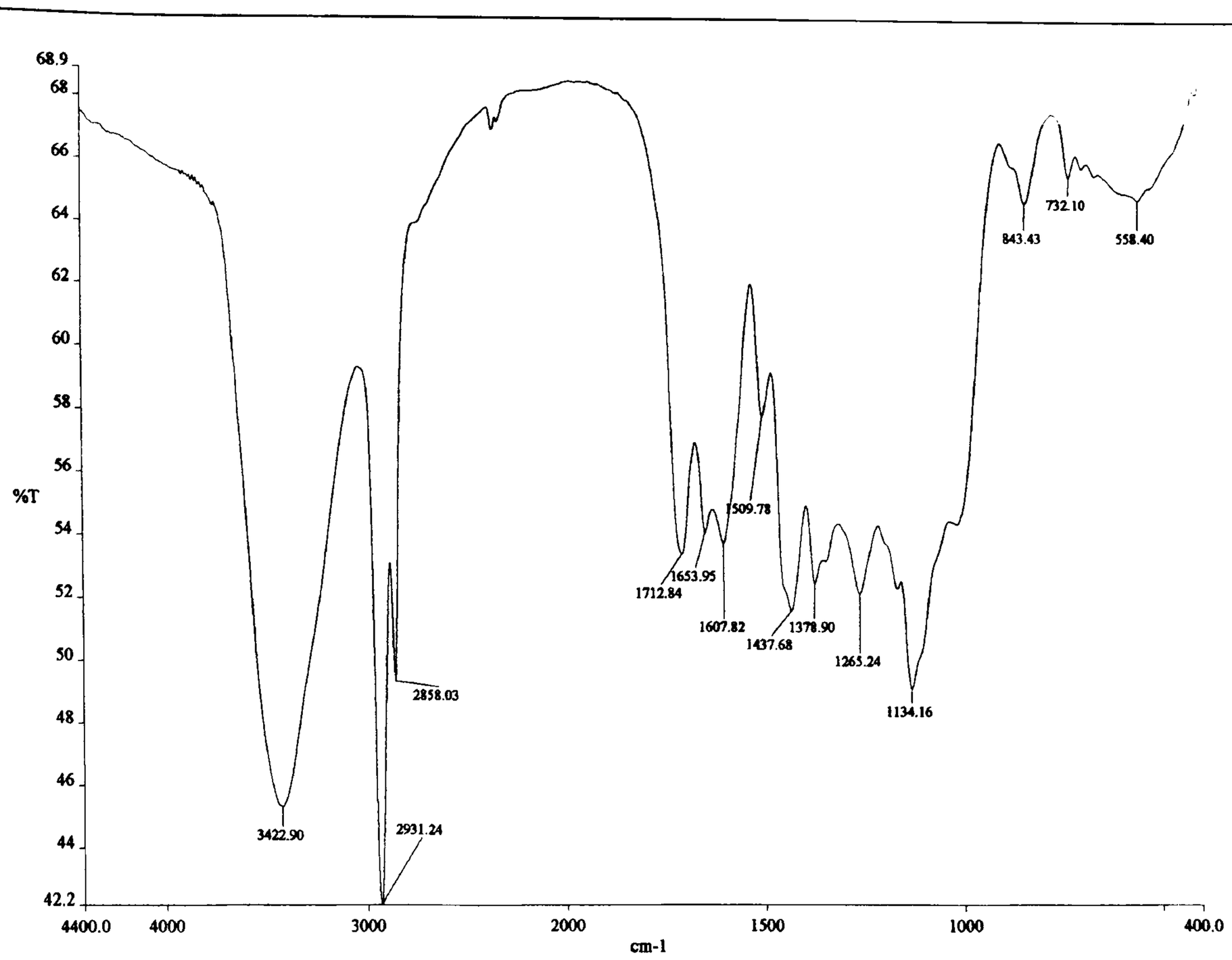
IR 14 Sporopollenin derivatised with (a) PCl_5 and (b) $SOCl_2$.



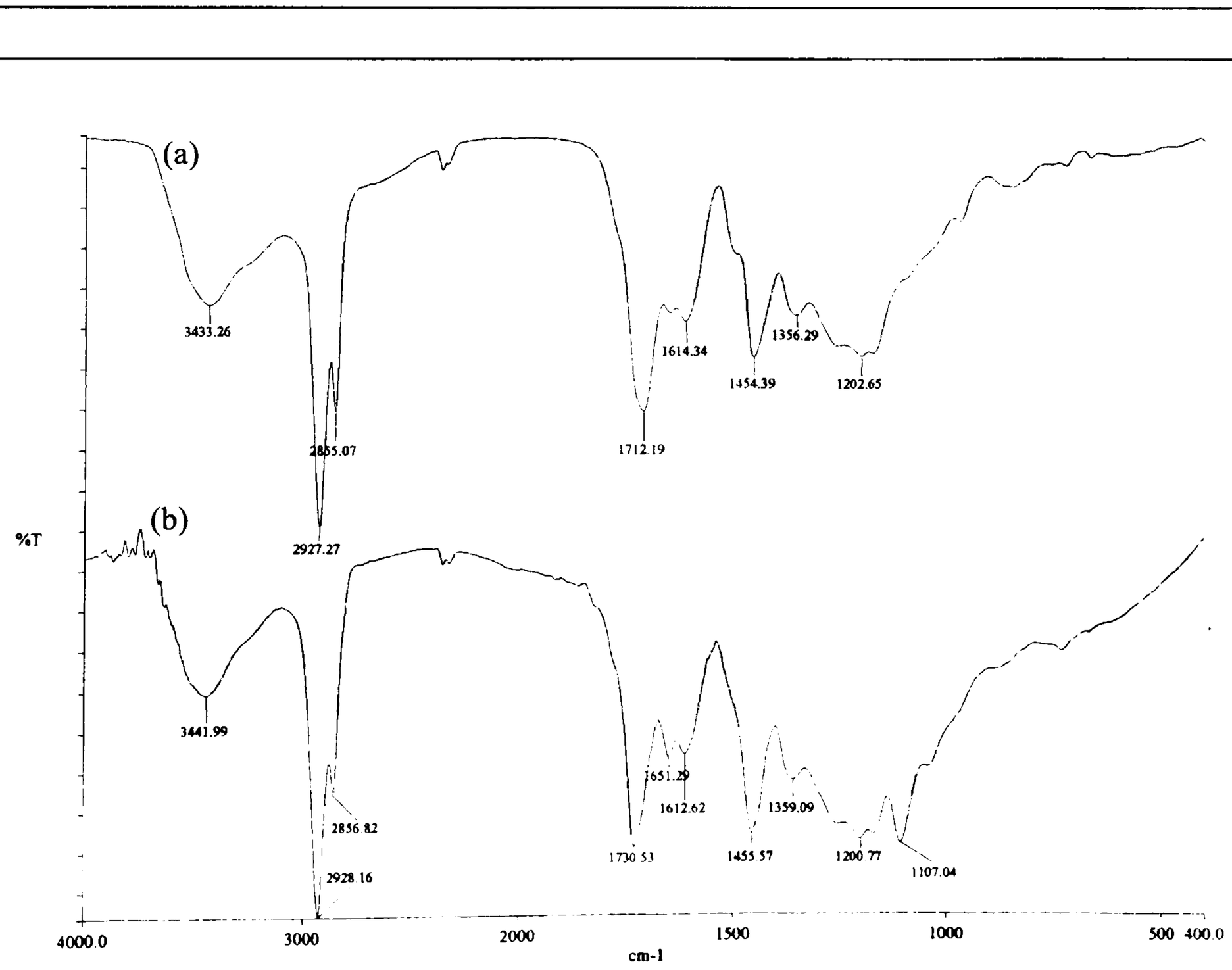
IR 15 (a) Acetylated Sporopollenin, (b) Underivatised Sporopollenin.



IR 16 Sporopollenin reduced with $LiAlH_4$.



IR 17 Sporopollenin Reduced with $NaBH_4$.



IR 18 (a) Underivatised Sporopollenin, (b) Chloromethylated Sporopollenin.