

THE SYNTHESIS OF THREE CHELATING REAGENTS
DERIVED FROM CHOLIC ACID

TO GAIL

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DERIVED FROM CHOLIC ACID

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ABSTRACT

Three cholic acid derived chelating reagents were synthesized. They were synthesized by the attachment of existing chelating ligands onto the side chain of cholic acid via a spacer chain which connects cholic acid and chelate. The incorporation of the ligands EDTA, DTPA and DHPTA onto suitable cholic acid derivatives was performed to produce the respective cholic acid derivatives: cholic acid-EDTA, cholic acid-DTPA, and cholic acid-DHPTA. The synthesis of a fourth chelating reagent was attempted by attaching HEDTA onto the cholic acid side chain but was unsuccessful. A complete account of the three syntheses and one partial synthesis are outlined in this thesis.

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List of Abbreviations

ESR	electron spin resonance
NMR	nuclear magnetic resonance
PAC	perturbed angular correlation
EDTA	ethylenediamine tetraacetic acid
HEDTA	N-hydroxyethyl ethylenediamine triacetic acid
DHPTA	diamino-2-hydroxypropane tetraacetic acid
DTPA	diethylenetriamine pentaacetic acid
DMAP	N,N-dimethylaminopyridine
BOC	t-butyloxycarbonyl
LAH	lithium aluminum hydride
trityl	triphenylmethyl
DMSO	dimethyl sulphoxide
DMF	N,N-dimethylformamide
EDTADA	EDTA dianhydride
THF	tetrahydrofuran
EDTAMA	EDTA monoanhydride
IR	infrared
DTPADA	DTPA dianhydride
DHPTME	DHPTA tetramethyl ester
TLC	thin layer chromatography
DCC	dicyclohexylcarbodiimide
UV	ultra violet

INTRODUCTION

1.1 Biological Probes

The use of naturally occurring compounds as probes for biological systems is of great importance in modern biochemistry and medicine. Many new compounds are being developed and are useful in performing various functions in biological systems such as the elucidation of metabolic processes (Jenkins et al.,1984), determination of microscopic structures (Yeh and Meares,1979) and tissue visualization (Scheinberg et al.,1982) through imaging techniques. These biological probes are of particular interest to the organic chemist as they are often derived from the synthetic modification of existing molecules found naturally in biological systems. The general strategy and synthesis of such compounds will form the basis of this thesis.

To be useful as a probe, the compound of interest must be detectable in the body and therefore must possess a particular spectroscopic property that can be conveniently monitored in vivo. Many metals exhibit interesting spectroscopic characteristics and therefore have potential as biological probes. For example, paramagnetic metals can be monitored in vivo by electron spin resonance (ESR) (Yeh and Meares,1979) and also have significant influence on spin

relaxation times of neighbouring nuclei which makes them useful for nuclear magnetic resonance (NMR) studies. Radioactive metals can be monitored using perturbed angular correlation (PAC) techniques (Sundberg et al., 1974) and metals such as some of the lanthanides exhibit fluorescent behaviour and can be detected with luminescence techniques (Leung and Meares, 1977). There are a large number of metals available for use; most of them (or their isotopes) exhibiting one or more of these spectroscopic properties. Their potential as biological spectroscopic probes is well substantiated, but many other factors must also be considered for their use.

The major problem associated with the use of metals in biological systems is their toxicity to the host organism; toxicity levels are usually well below concentrations required for in vivo spectroscopic studies. Strongly chelated metal ions however are suitable and are much less toxic to the host organism. To ensure metals remain chelated in vivo the chelating ligands must form chelate complexes which have high stability constants and are kinetically inert. Many polycarboxylate ligands such as ethylene diamine tetraacetic acid (EDTA) and its various analogues fit this criterion and are therefore quite suitable for use in biological systems. Their use has been documented previously in the literature. Many in vivo experimental studies have been cited including the study of

metabolism, cell membrane structural determination, and tissue visualization.

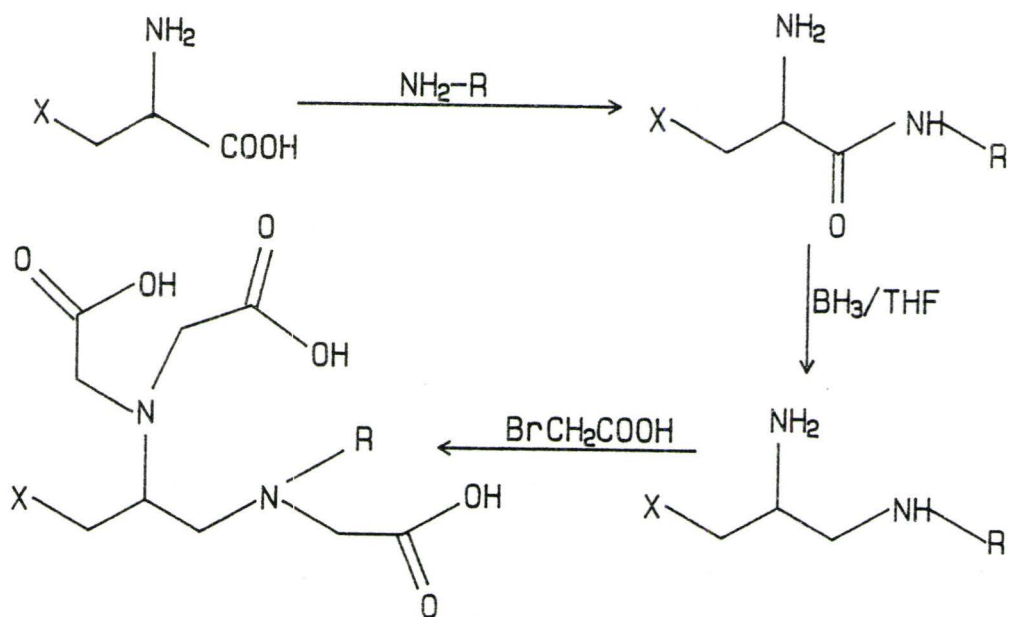
For the study of a particular tissue or specific region of the body it would be advantageous to be able to localize the chelated metal ion to the site of interest. In doing so, higher metal ion concentrations would be present in the area of interest and would therefore enhance the spectroscopic effects. The use of metal chelates covalently attached to naturally occurring molecules which are specific for cell or tissue receptor sites or have particular metabolic roles would be very useful and would selectively localize the metal ions to the biological areas of interest. A means of attaching the chelate to the tissue specific molecule is therefore required. Previous attempts have been successful (Meares and Wensel, 1984; Hertzberg and Dervan, 1984; Takeshita, 1982) and some of the strategies are outlined in the following section.

The objective of this project is to devise and carry out a synthesis of a tissue specific chelate (bifunctional chelate) which would have potential as an image enhancer or as a biological probe. More specifically this project deals with the attachment of "EDTA like" chelating moieties to the naturally occurring steroid, cholic acid (1), in such a way that it may have potential use as a probe in intestinal studies (Runge et al., 1984).

1.2 Review of Previous Syntheses

One of the first reports of the synthesis of a "bifunctional chelating reagent" was in 1974 (Meares et al., 1974). The authors had prepared azoproteins labelled with In(III) for the detection and localization of tumors. The synthesis was lengthy however, and they improved upon it in 1979 (Meares et al., 1979). Common amino acids were employed as starting materials and the use of a variable "R" group allowed incorporation of various side chains in which to develop different chelating moieties (see Scheme 1.1). The "X" group represents the side chain of the amino acid which is used as the link between the biological molecule and

Scheme 1.1

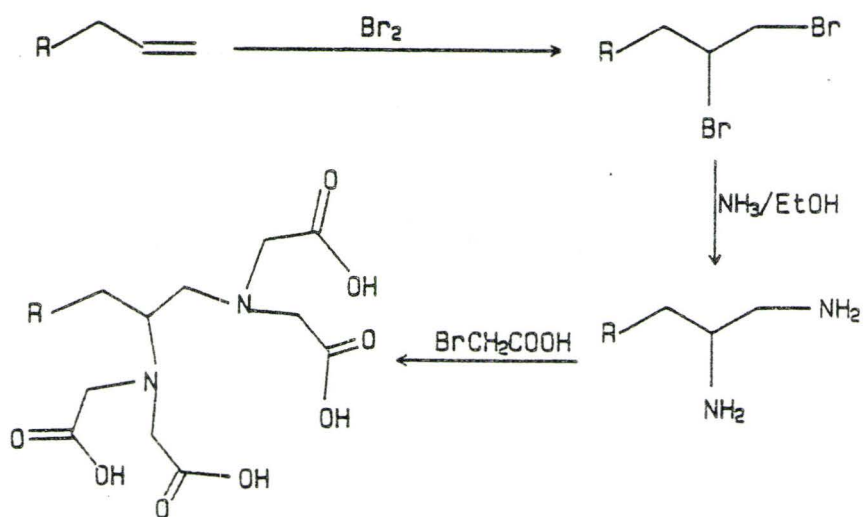


chelating moiety. The synthesis is very versatile as it enables the use of many naturally occurring amino acids as starting materials, and therefore provides for the synthesis of a potentially large number of biological chelates. The chelating portion of the molecule is developed stepwise throughout the synthesis as opposed to using the chelate intact as a starting material. This will be referred to as a "total synthesis approach".

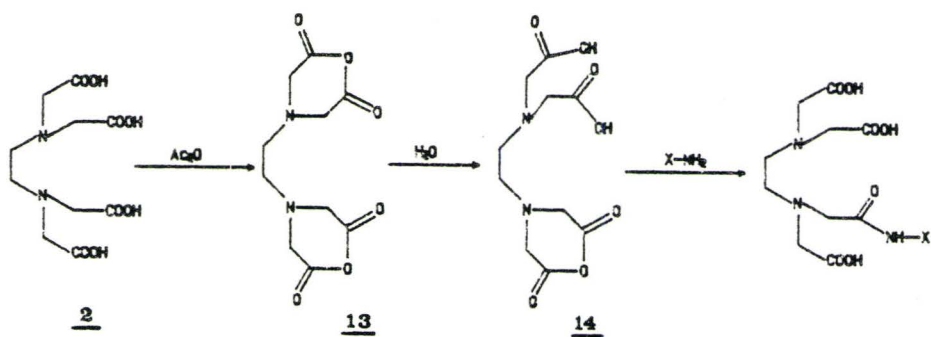
In 1976 another total synthesis approach was reported by Takeshita (Takeshita et al., 1976). The authors were developing dispersing and emulsifying agents by synthesizing chelates with long alkyl chains attached. The methodology however, could be applied to the synthesis of chelates for biological systems. The starting material in this case was a long chain alkene with the double bond at the terminal carbon. The alkene was brominated and then the bromines displaced with ammonia to form an ethylene diamine backbone with an alkyl chain attached at one of the ethylene carbons. The final step, as in Meares' case, was to fully substitute the amines with acetic acid residues to give the polycarboxylate ligand as shown in Scheme 1.2.

Four years later in the same laboratory a new synthetic scheme was introduced (Takeshita et al, 1982). Again their goal was to synthesize long chain alkyl chelates but their methodology was even more applicable to the synthesis of a biological chelate. The starting material

Scheme 1.2

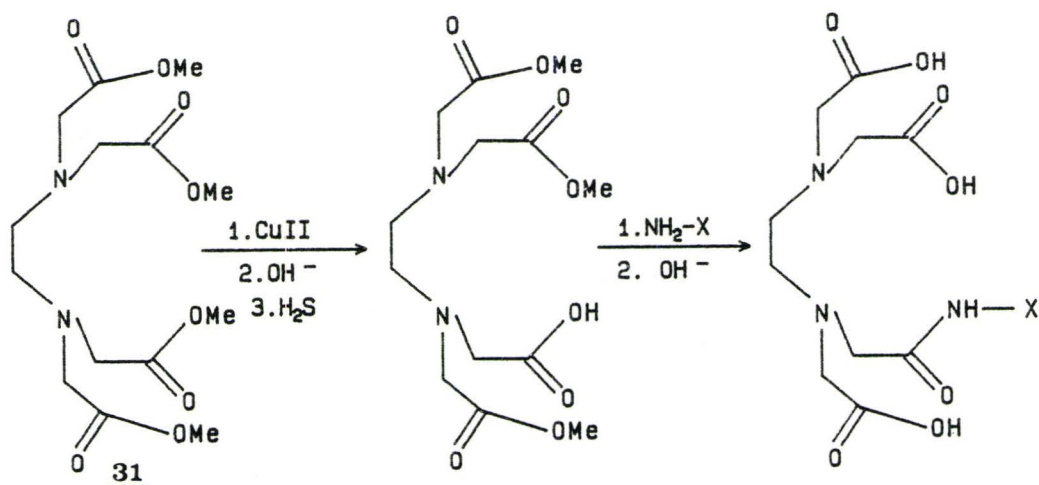


Scheme 1.3



was a primary alkyl amine which was preferable to the previous case since primary amines are abundant (eg. proteins) in biological systems. The synthesis was concise and yields were excellent. The chelating moiety was different from the two previously discussed cases because one carboxyl group was converted to an amide (see Scheme 1.3). Evidence however, showed the tricarboxy chelate to have good chelating properties (Takeshita et al., 1982). The procedure had many good qualities, most important of which was convenience. It utilized EDTA as a starting material, and thus eliminated the need for its synthesis as in the total synthesis approach. The synthesis was also versatile as EDTA was converted to the monocyclic anhydride which is a highly reactive compound and can be used as an EDTA coupling reagent for any number of biological molecules possessing a free amine function.

Scheme 1.4



One of the more recent attempts (Hertzberg and Dervan, 1984) utilizes a totally different technique to produce a final product with the identical chelating moiety as in Takeshita's later synthesis (1982) as shown in Scheme 1.4. The synthesis was not as concise but yields were good. EDTA again was used as a starting material. It was converted to the tetraester and chelated to Cu(II). Hydrolysis of one ester followed by precipitation of the copper using hydrogen sulphide gas left the triester. The free carboxyl group was then activated using standard coupling reagents, the biological amine was added to form the amide, and the remaining esters removed by hydrolysis.

Of the four previous strategies discussed, two methods in particular show promise. Meares' synthesis (1976) shows potential for its versatility as it provides for the development of different chelating moieties attached to the naturally occurring starting material. Takeshita's synthesis (1982) is also efficient and shows great versatility in its use of an activated EDTA unit suitable for attachment to a variety of biological amines. These two strategies as well as two new synthetic strategies have been used in the attachment of chelating moieties to cholic acid and will be discussed throughout this thesis.

1.3 Cholic Acid and the Chelating Moieties

1.3.1 Cholic Acid

Cholic acid (1) is a trihydroxy bile acid that is found naturally in the intestinal tract of many animals. It exists exclusively as salts of amino acid conjugates (usually of glycine and taurine) in which it is joined to the amino acid via an amide link (see Fig 1.1c). These conjugates are amphipathic in nature and exist mainly as micelles in solution. Their purpose in the intestine is to solubilize lipids by acting as emulsifying agents or biological soaps, and they are absorbed through the lower intestinal tract lining via recognition sites along the lining surface.

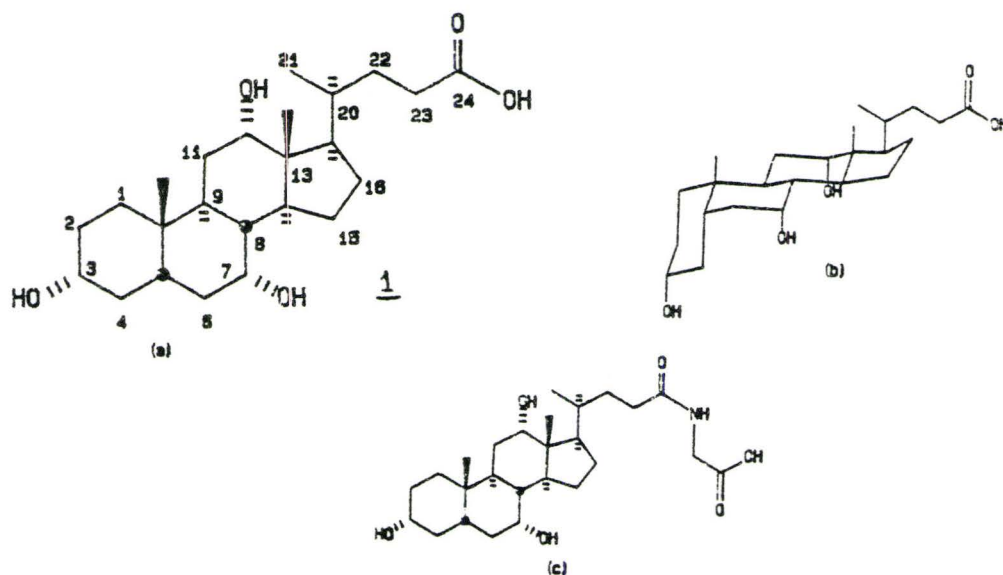


Fig 1.1 Cholic acid depicting a) its structure and numbering system b) side view of the steroidal ring system c) an example of its natural form as the glycine conjugate.

Cholic acid has been chosen for experimentation for two reasons. It is found naturally in an isolated area in the body (tissue selective) and is therefore potentially useful as a biological probe for that specific area. Also, it belongs to the "steroid" class of molecules and can therefore be used as a model for the attachment of chelates to a variety of other related steroids.

1.3.2 Chelating Moieties

Four chelating moieties have been used in this research. All are polycarboxylate ligands based on variations in the structure of EDTA (2), and are therefore expected to have good chelating properties.

EDTA itself has been examined. It is the most popular of the chelating reagents and has been used

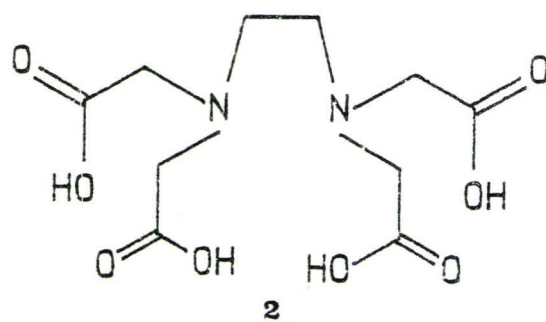


Fig 1.2 Ethylene diamine tetraacetic acid (EDTA)

extensively in analytical chemistry. It is a hexadentate ligand and is therefore ideal for the co-ordination of metal ions in an octahedral geometry. It has high stability constants when chelated to many metal ions (Pribil,1972).

Closely related to EDTA is N-(hydroxyethyl) ethylenediamine triacetic acid (HEDTA)(3). It is identical to EDTA except it has a hydroxyl group replacing one carboxylic acid group. The hydroxyl group is expected to be only weakly co-ordinating and therefore HEDTA is considered to be pentadentate.

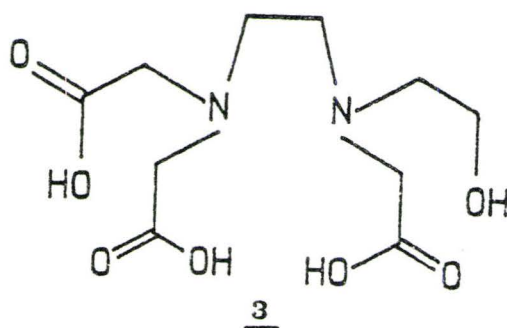


Fig 1.3 N-(hydroxyethyl) ethylene diamine tetraacetic acid (HEDTA)

Another compound closely related to EDTA is 1,3-diamino-2-hydroxy propane tetraacetic acid (DHPTA)(4). This ligand has a three carbon diamine backbone with a hydroxyl group on the middle carbon. It also is hexadentate and has been shown to have good chelating properties. The

hydroxyl group is not directly involved in chelation but may aid in solubilizing the chelate or can be used for derivatization as is the case in this research.

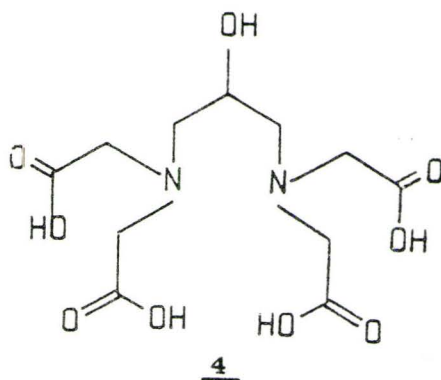


Fig 1.4 1,3-diamino-2-hydroxypropane tetraacetic acid (DHPTA)

The fourth chelating moiety to be discussed is an extension of EDTA in which the ethylene diamine backbone is replaced with a diethylene triamine backbone where each nitrogen is fully substituted with acetic acid residues.

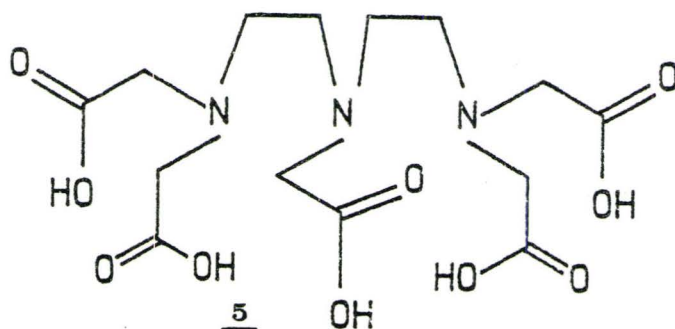


Fig 1.5 Diethylene triamine pentaacetic acid (DTPA)

Diethylene triamine pentaacetic acid (DTPA)(5) is an octadentate ligand and is especially useful for the co-ordination of larger metal ions. It also has been shown to have high stability constants when chelated to many metal ions.

1.4 Strategies and Specific Objectives

The specific objective of this project is to attach the four aforementioned chelating ligands onto cholic acid to form potentially useful intestinal probes. There are two criteria for the attachment of chelating moieties to cholic acid. First, it must be done in such a manner that it does not affect the tissue specificity of cholic acid, and secondly, the chelate must be introduced in such a way that it retains good chelating qualities.

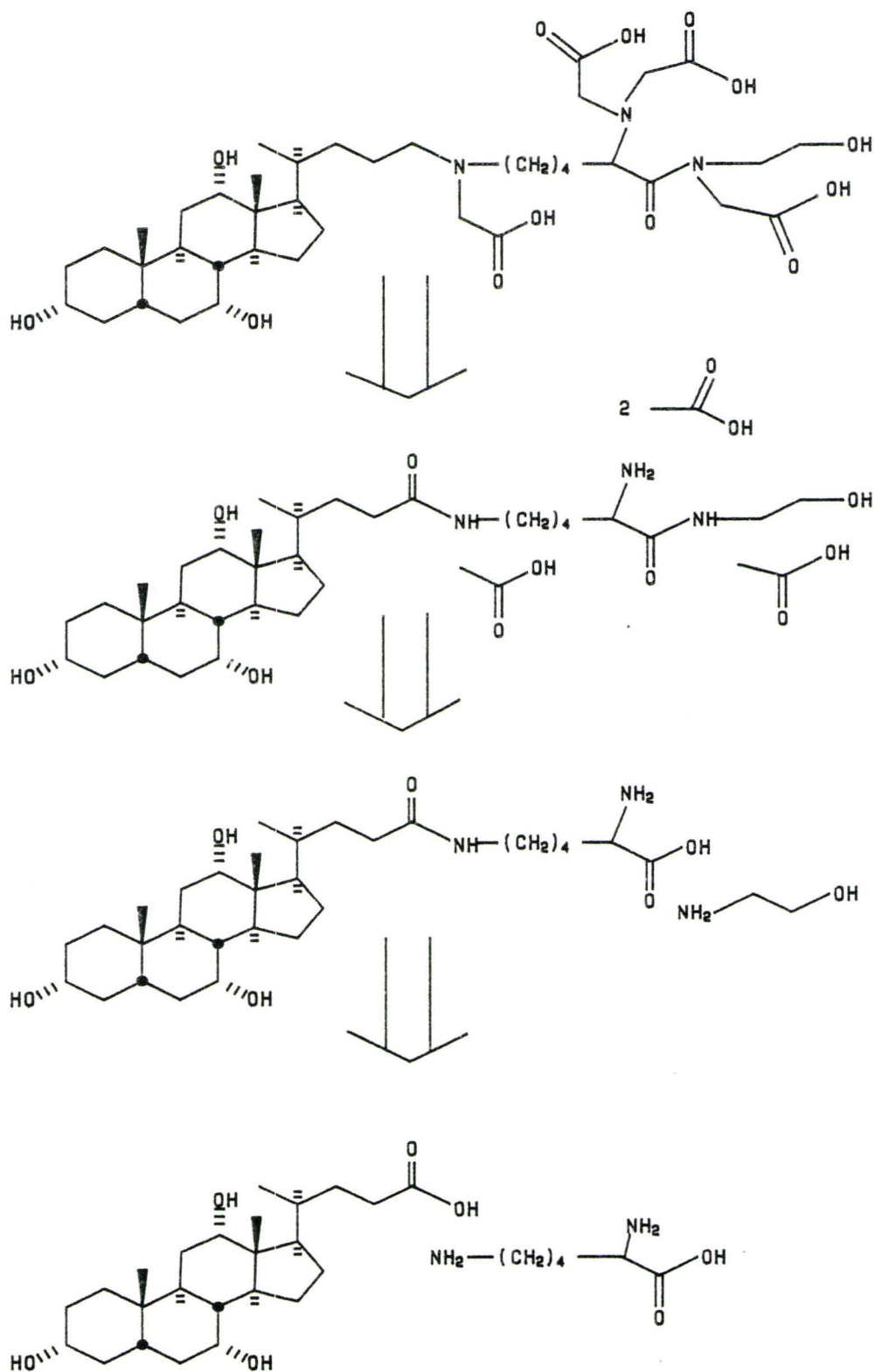
It is thought that the cyclopentanoperhydrophenanthrene ring system of cholic acid is the portion of the molecule which provides its tissue specificity, either by hydrophilic interactions from the hydroxylated α face, or through hydrophobic interactions from the hydrocarbon β face. The free side chain which exists as variable amino acid conjugates in nature is not thought to play a role in tissue specificity and would be the least likely site to prevent tissue recognition if it was chemically altered. It is logical therefore to attach the chelate to cholic acid at the free carboxyl site. The use of a diamine spacer chain to

form an amide linkage at the cholic acid carboxyl group would act to mimic the amino acid which is normally present in nature. It would also provide adequate space between the steroid and chelating moiety to ensure there are no steric interactions between the two which may hinder the steroids tissue recognition ability or affect chelating ability.

There are two ways to link the chelating ligand to the spacer chain. Using EDTA as a typical chelating moiety (see Fig 1.2) it can be seen that functionalization can occur at a carbon atom (most likely through the ethylene diamine backbone) or through attachment at a carboxyl group. Both strategies have been considered and are dealt with separately.

The first synthesis to be considered is a total synthesis approach where the chelating moiety HEDTA is linked to cholic acid through a spacer chain attached to a carbon in its ethylene diamine backbone. The chelating moiety is developed as in Meares' synthesis (see Scheme 1.1) and attachment of the chelating moiety to the steroid is achieved using lysine as the spacer chain. The ϵ -amino group is used to form an amide link to cholic acid and the α -amino group becomes available for incorporation into the ethylene diamine backbone. Ethanolamine is used to form an amide at the new carboxyl site. This introduces the second nitrogen in the ethylene diamine backbone and also introduces the free hydroxyl side chain in the chelate. The initial

Fig 1.6 Retrosynthetic scheme for the synthesis of Cholic acid-HEDTA



intention of the hydroxyl side chain was to have a weakly co-ordinating ligand such that rapid water exchange could occur with the chelated metal ion and therefore affect the water protons NMR relaxation times. Reduction of the amides and addition of the acetic acid residues leads to the final chelate product; cholic acid-HEDTA (6).

The following three syntheses are examples of chelate attachment through a functional group and all use the chelating moieties as starting materials. DHPTA will be functionalized at the hydroxyl site and EDTA and DTPA will be functionalized at a carboxylic acid site. Functionalizing the chelates in this manner implies the chelating ligands will have varying co-ordination numbers (Fig 1.7). EDTA and

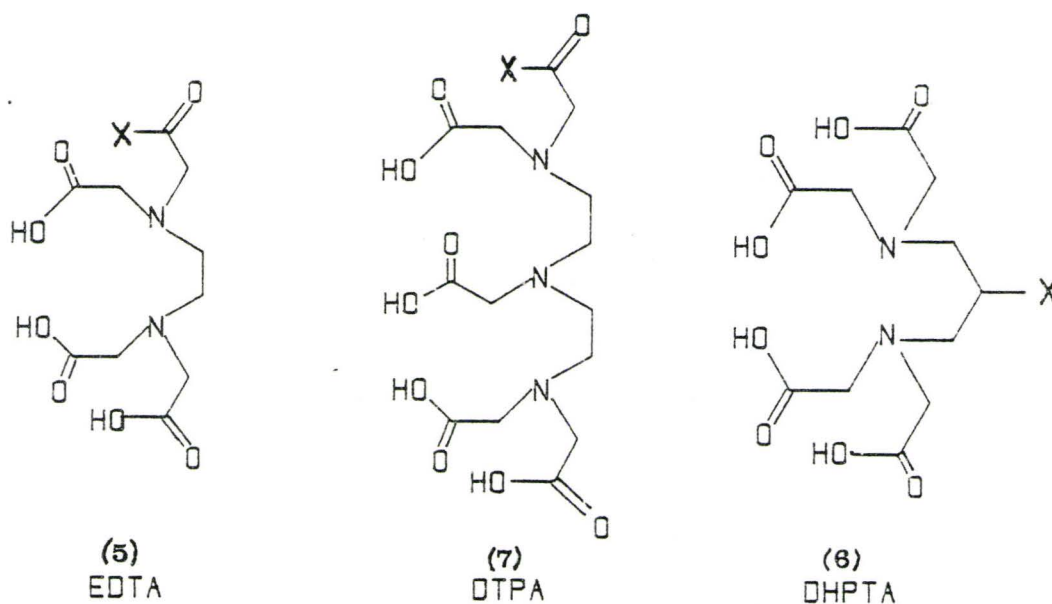


Fig 1.7 Co-ordination numbers (shown in brackets) of EDTA, DTPA, and DHPTA chelating derivatives.

Fig 1.8 Retrosynthetic scheme for the synthesis of Cholic acid-EDTA

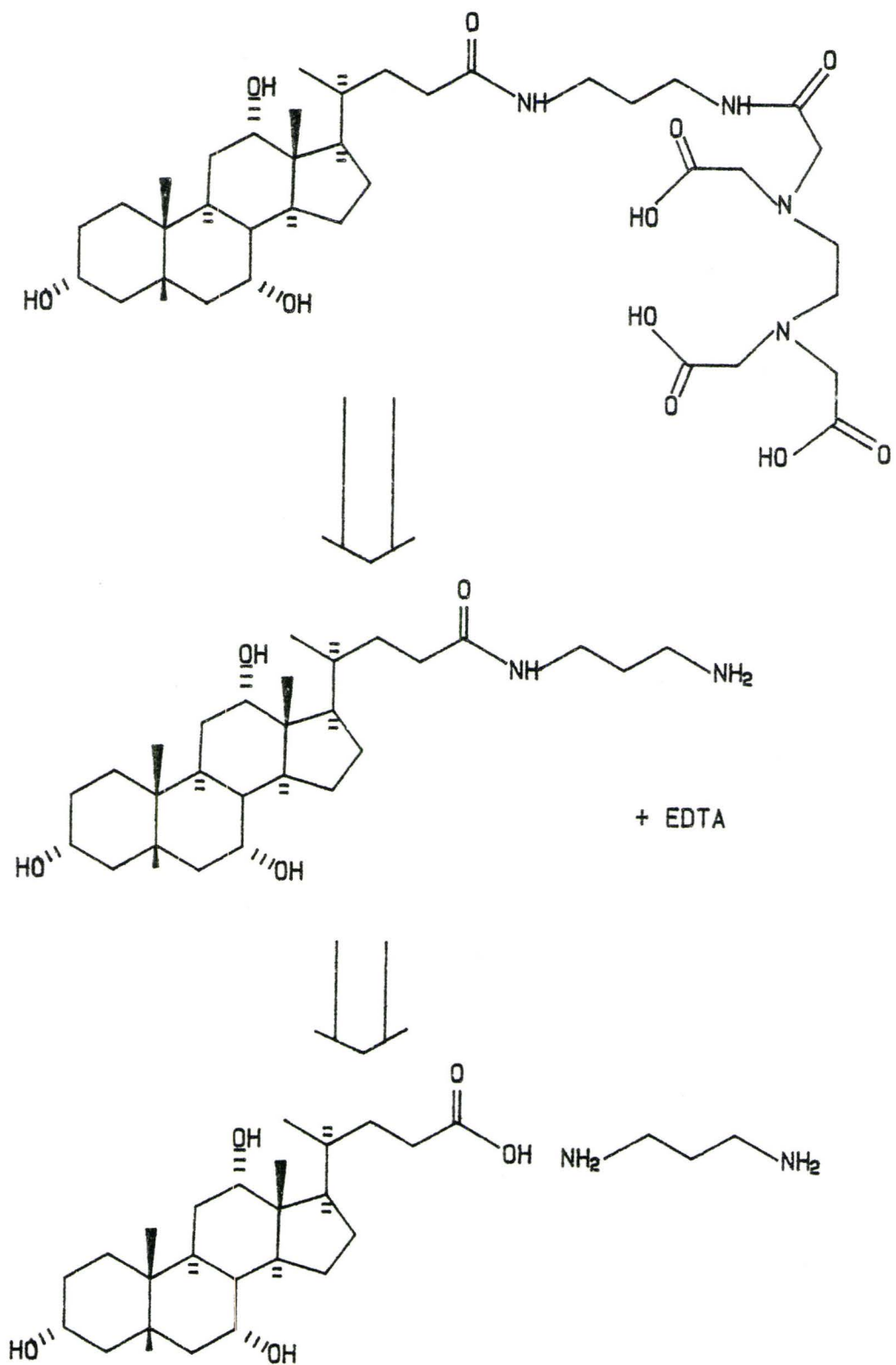


Fig 1.9 Retrosynthetic scheme for the synthesis of Cholic acid-DPTA

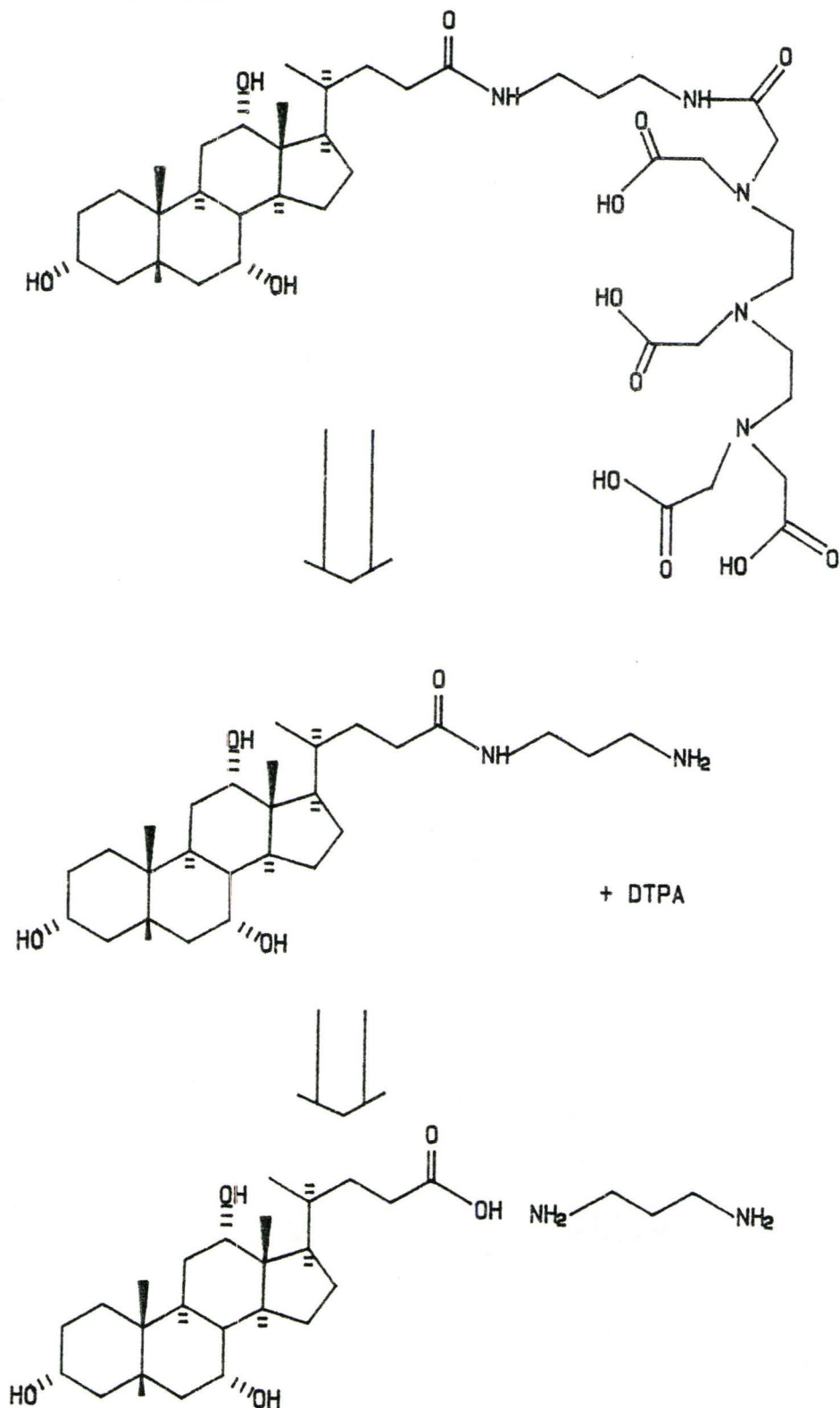
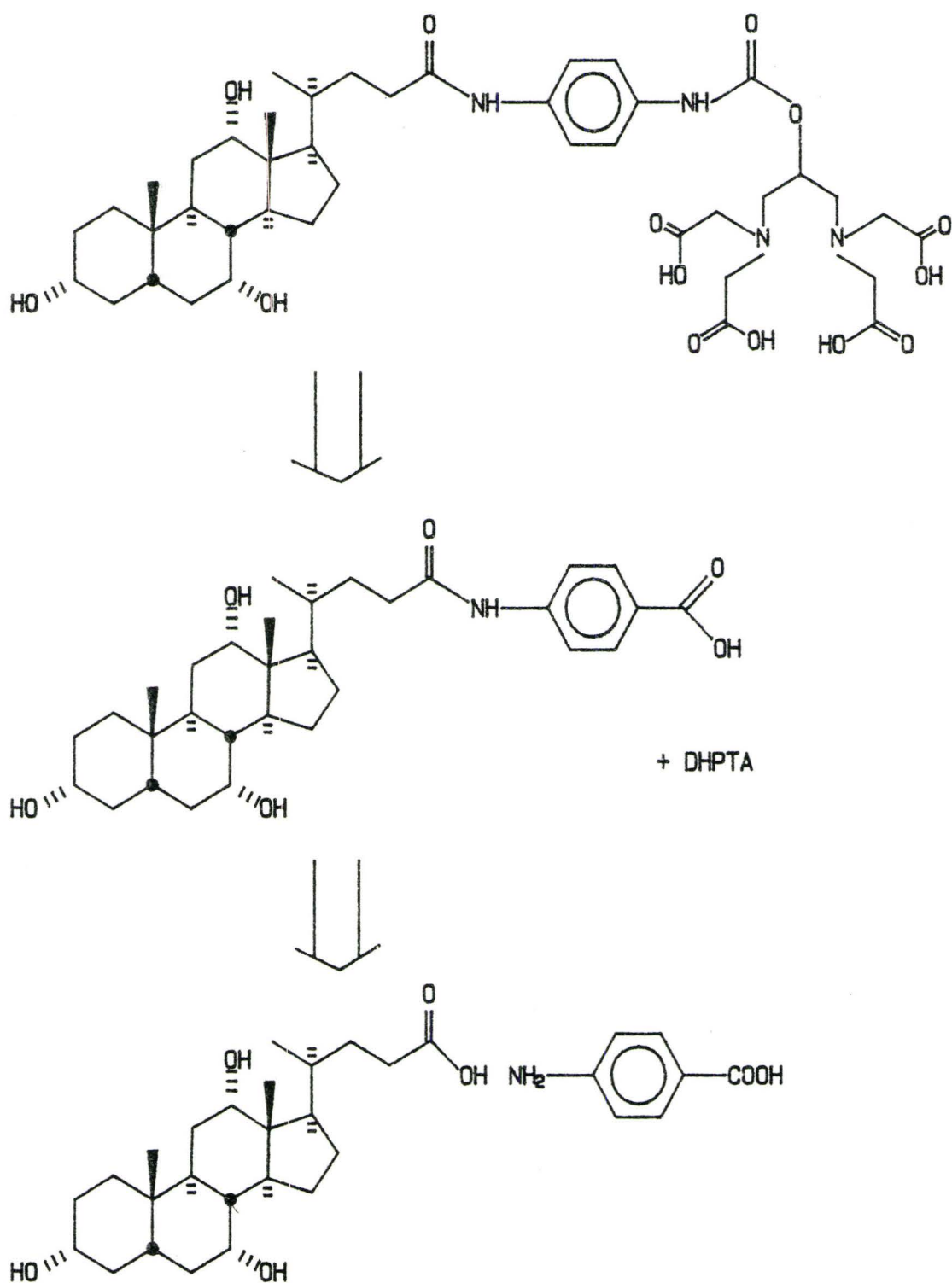


Fig 1.10 Retrosynthetic scheme for the synthesis of Cholic acid-DHPTA



DTPA with one carboxyl site functionalized become penta- and hepta-dentate, respectively, and DHPTA remains hexa-dentate. The different ligand co-ordinating capabilities could be potentially useful in the co-ordination of different metal ions in solution.

For the attachment of EDTA and DTPA to cholic acid a spacer chain is required that will form an amide with cholic acid (as in naturally occurring amino acid conjugates) and also form a stable linkage to the carboxyl group of the chelate. An amide linkage would also be favorable here and therefore the use of a diamine spacer chain would be appropriate. Attachment of the diamine spacer chain to cholic acid at one end of the diamine leaves a primary amine for amide formation with the chelate (EDTA or DTPA). Appropriate monocarboxyl activation of the chelates followed by reaction with the steroidal amine produces the final chelates: cholic acid-EDTA (7) and cholic acid-DTPA (8) as shown in Fig 1.8 and Fig 1.9.

The attachment of DHPTA to cholic acid is to be carried out by linking the spacer chain attached to cholic acid to the hydroxyl group of DHPTA. The spacer chain in this case is p-diaminobenzene and reasons for its employment rather than an alkyl diamine will become apparent later in this report. The actual synthetic reagent for the diamine spacer chain is p-amino benzoic acid. Amide formation with the cholic acid leaves an aromatic carboxyl group available

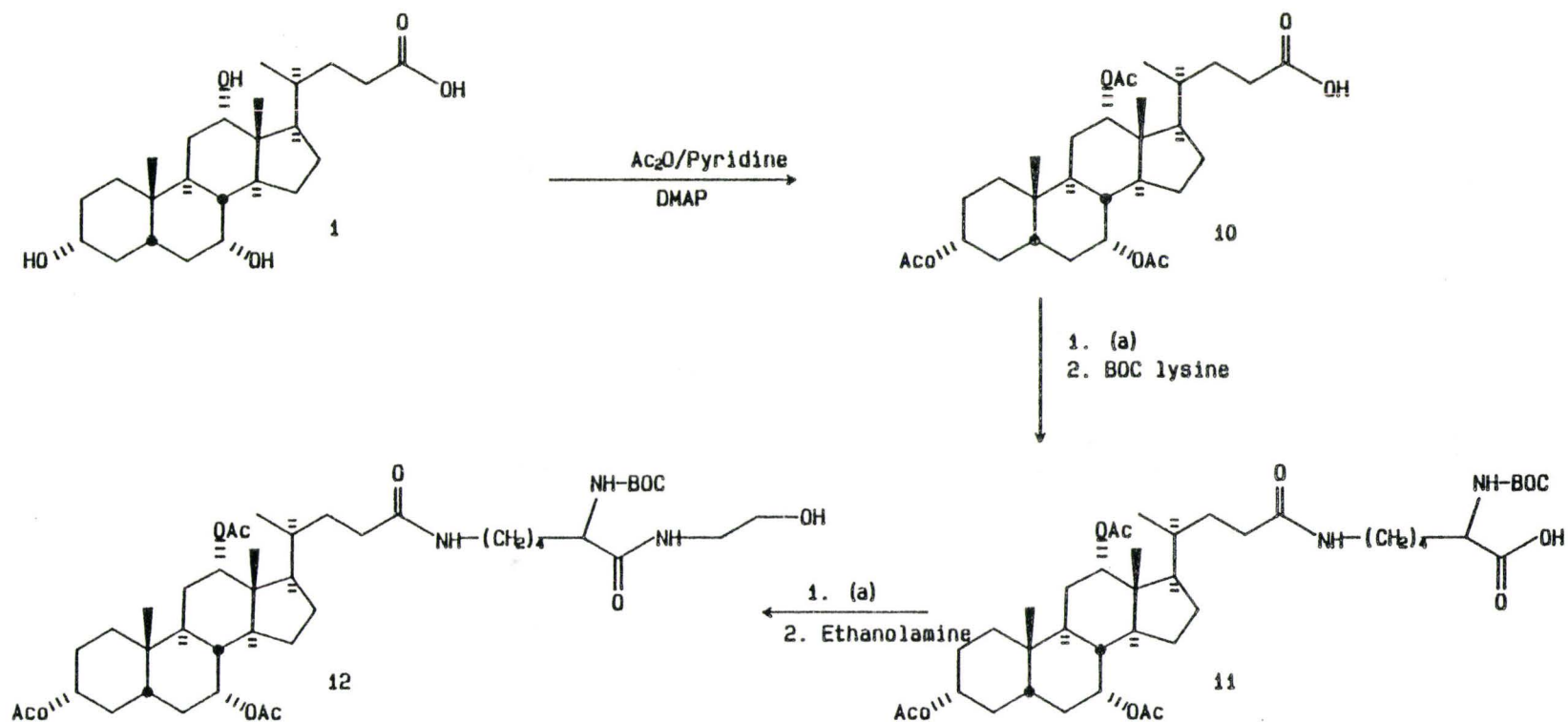
for linkage to the chelate. Functional group manipulation of the carboxyl group to form the isocyanate allows carbamate formation and a strong chelate linkage, producing cholic acid-DHPTA (9).

It should be noted that the strategies dealt with in this section are general, and many manipulations such as protecting and deprotecting steps have been omitted. Detailed strategic analysis of each synthesis appears in the discussion section separately under the heading of each specific compound.

RESULTS

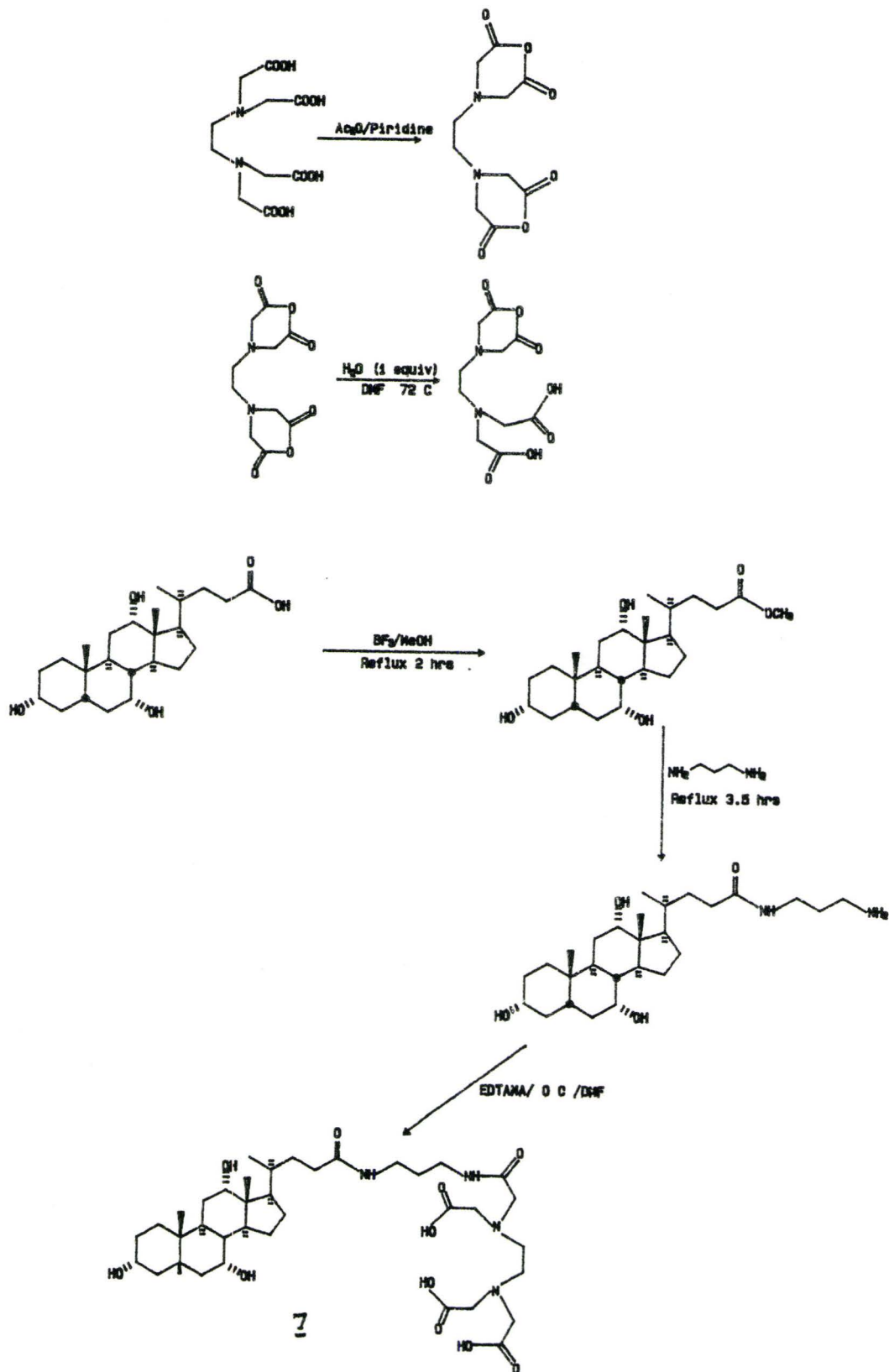
Three chelating reagents derived from cholic acid were synthesized. The synthesis of a fourth derivative was attempted but was unsuccessful. The reaction schemes used in the three syntheses and the one partial synthesis are reported in Schemes 2.1 to 2.4.

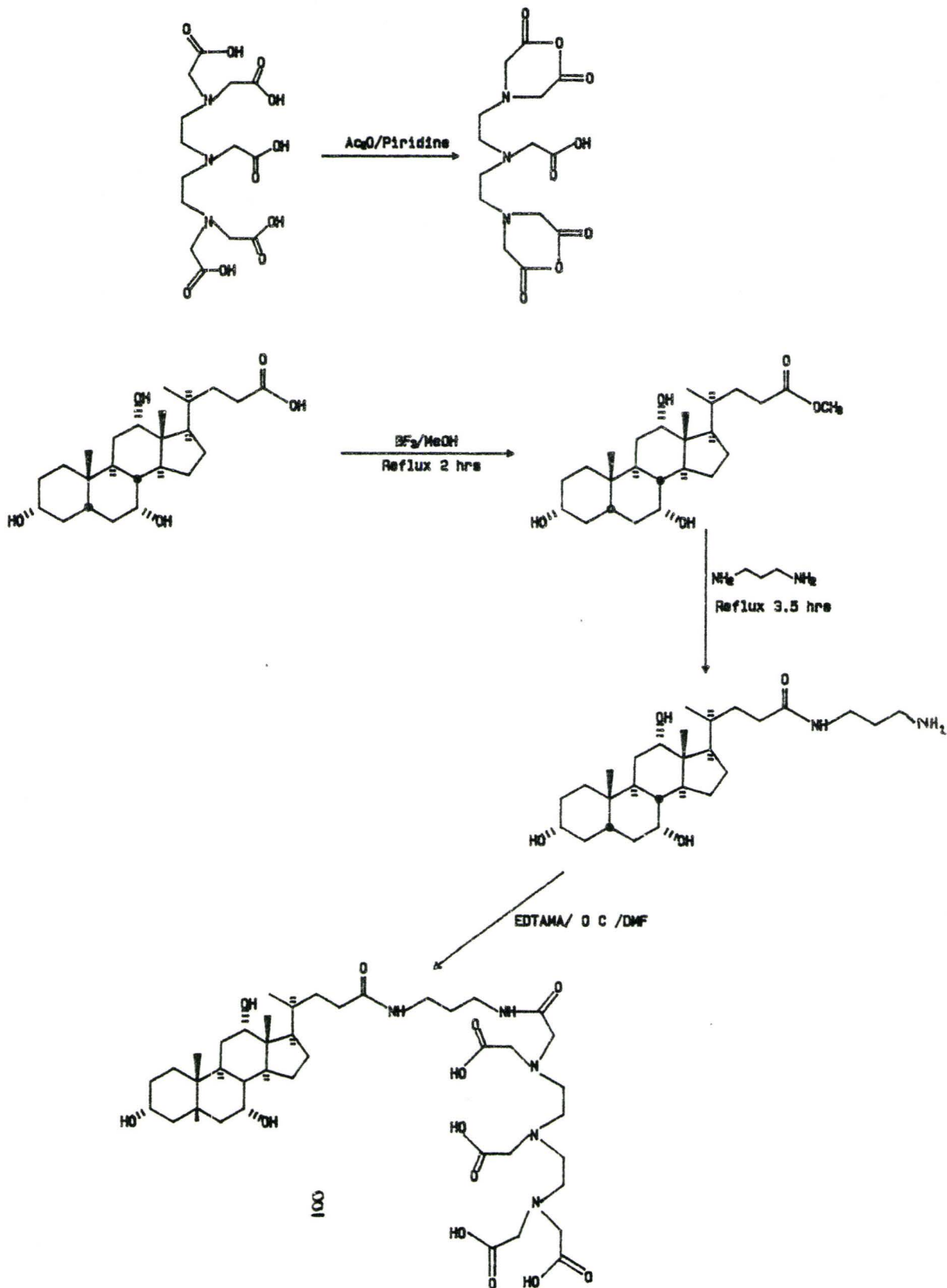
Scheme 2.1 Partial synthesis of Cholic acid-HEDTA



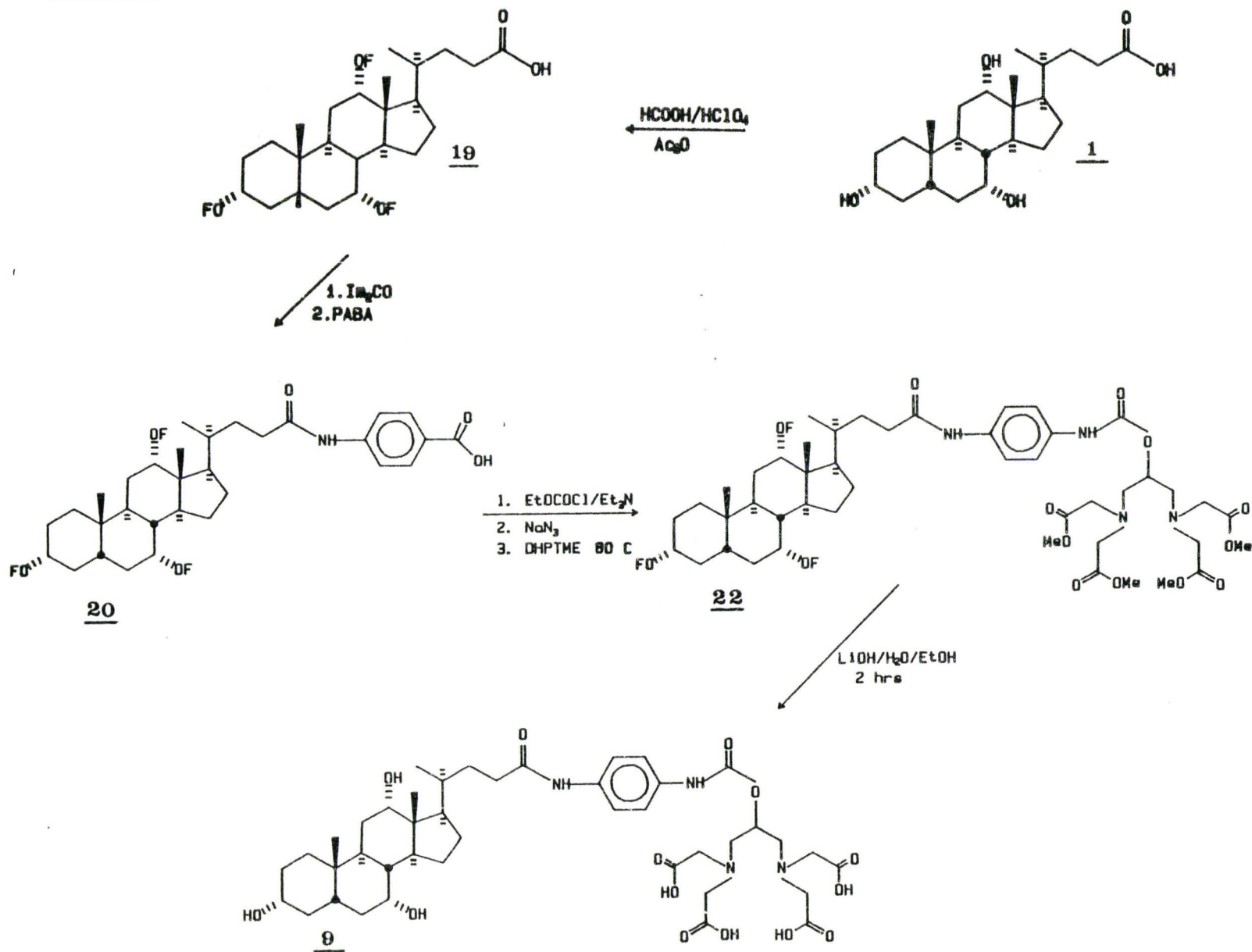
(a) = isobutyl chloroformate / triethyl amine (-20 C)

Scheme 2.2 Synthesis of Cholic acid-EDTA





Scheme 2.4 Synthesis of Cholic acid-DHPTA



DISCUSSION

3.1 TOTAL SYNTHESIS APPROACH OF CHOLIC ACID-HEDTA

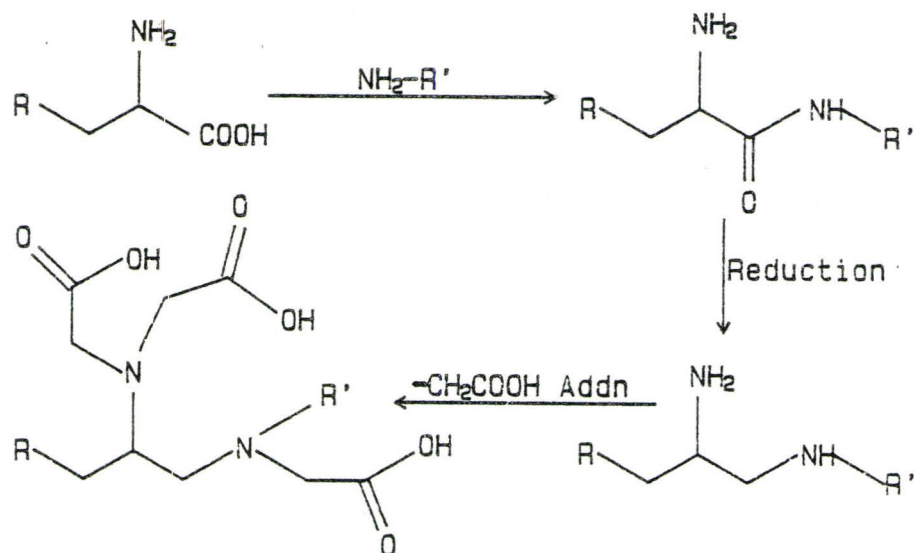
3.1.1 Synthetic Strategy

The synthetic strategy used in this synthesis follows closely that used previously (Meares and Wensel, 1984). The chelating moiety (something resembling EDTA) is to be attached to the biological molecule through a carbon atom in the ethylene diamine backbone and the attachment is to be achieved in a total synthesis approach. This means the chelating moiety will be developed throughout the course of the synthesis rather than being used intact as a starting material.

Amino acid amides contain a two carbon fragment between nitrogens, one of which is doubly bonded to an oxygen and the other is bonded to the amino acid side chain. This framework can be transformed into a carbon substituted ethylene diamine unit by reduction of the amide carbonyl to a methylene. Addition of acetic acid residues to the amine functions produces an EDTA moiety which may be attached to a target molecule through the amino acid side chain. The use of a primary amide produces a tetracarboxy-chelate whereas the use of an N substituted amide gives rise to a tricarboxy-chelate with a variable side chain (R' in

Scheme 3.1) dependent on the N substitution. This is depicted diagrammatically in Scheme 3.1.

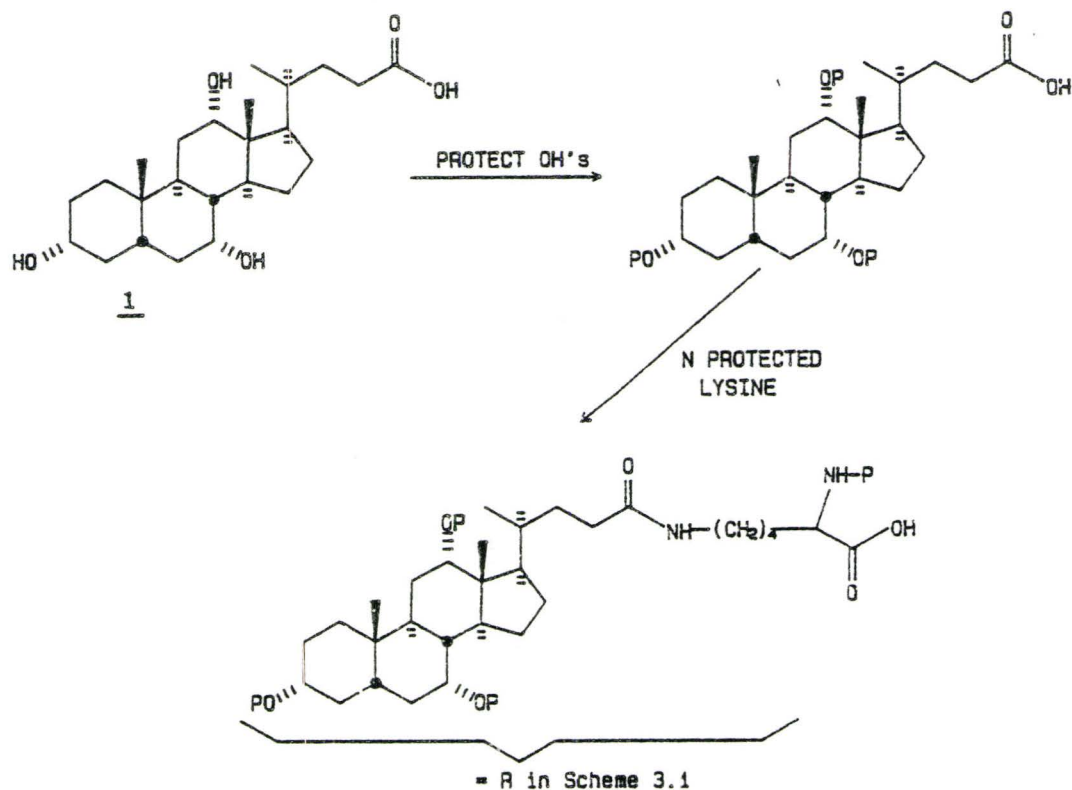
Scheme 3.1



To adapt this strategy to the synthesis of a cholic acid chelating derivative, cholic acid must therefore first be transformed into an amino acid. Alpha amino protected lysine would be useful for this purpose as an amide could be produced using the ϵ -amino group leaving the α -amino group free for later manipulation. Protection of the hydroxyl groups on cholic acid and formation of the amide with the α -protected lysine gives the protected version of the starting amino acid shown previously in Scheme 3.1. Formation of the second amide introduces the N-substituted variable side chain onto the chelating moiety. Deprotecting steps, followed by reduction of both amide carbonyls and addition of acetic acid residues produces the final cholic acid

chelate (see Scheme 3.2).

Scheme 3.2



3.1.2 Attempted Synthesis

Protection of the hydroxyl groups on cholic acid was performed by the preparation of cholic acid triacetate. Reaction of cholic acid with acetic anhydride in pyridine with dimethylaminopyridine (DMAP)(23) as an acetylating catalyst (Fieser and Fieser, 1972) produced 10 in 2 hrs. Without the use of DMAP the reaction was incomplete in 3 days under otherwise identical conditions. The mechanism

for DMAP catalysis is shown in Fig 3.1. The proton NMR spectrum showed the presence of three singlets at

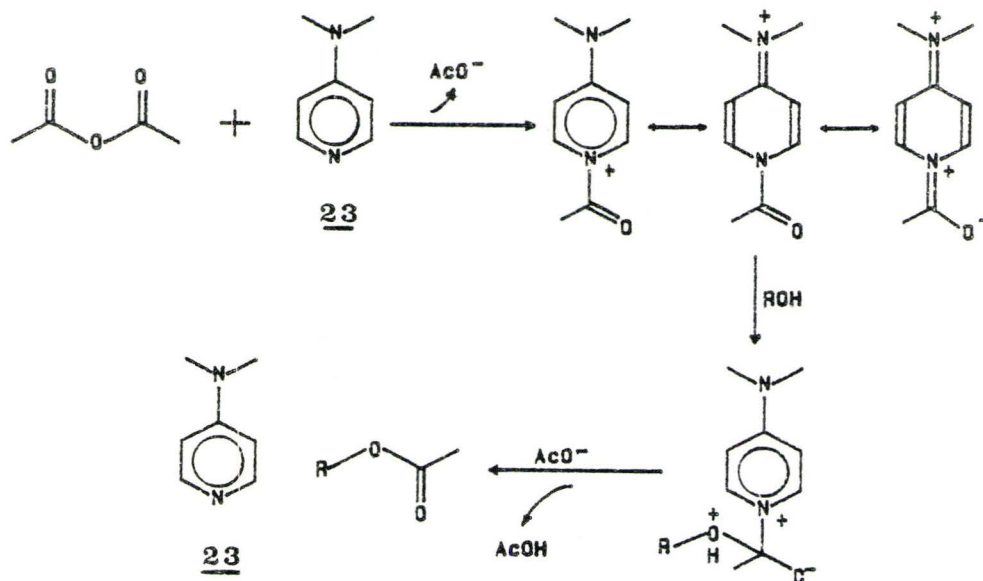


Fig 3.1 Mechanism of acetylation using dimethylaminopyridine as a catalyst.

approximately 2 ppm corresponding to the three acetate methyl groups as well as a downfield shift of the protons on the three acetylated carbons from (3.2-3.8) ppm to (4.5-5.0) ppm. The ammonia chemical ionization mass spectrum showed a $M+18$ peak at m/z 552 corresponding to $M+\text{NH}_4^+$.

Alpha-N-t-butyloxycarbonyl lysine (BOC-lysine) was attached to the protected cholic acid using the mixed anhydride method (Anderson et al., 1967) (isobutyl chloroformate and triethyl amine). The mixed carbonic

anhydrides are very reactive and reaction is carried out at very low temperatures, thus reducing other side reactions. The mechanism is shown in Fig 3.2. The signal of the t-butyl group of the product (11) was evident in the

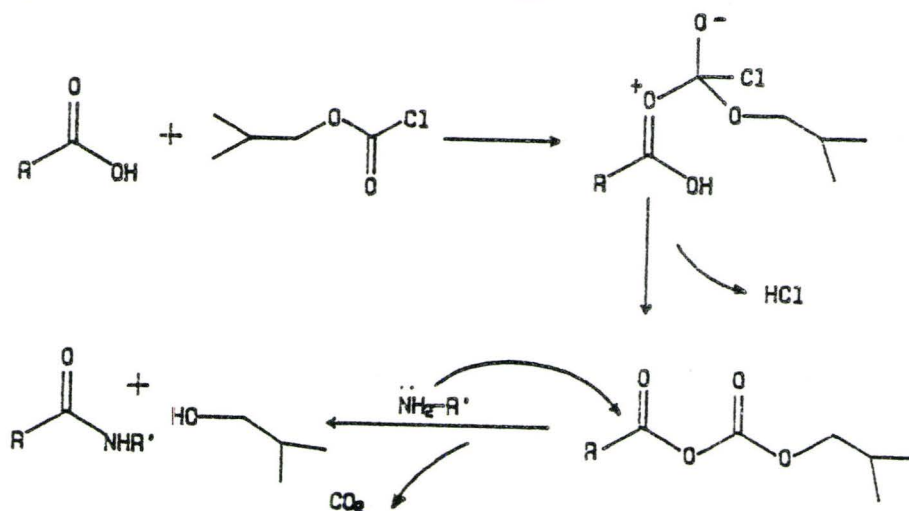
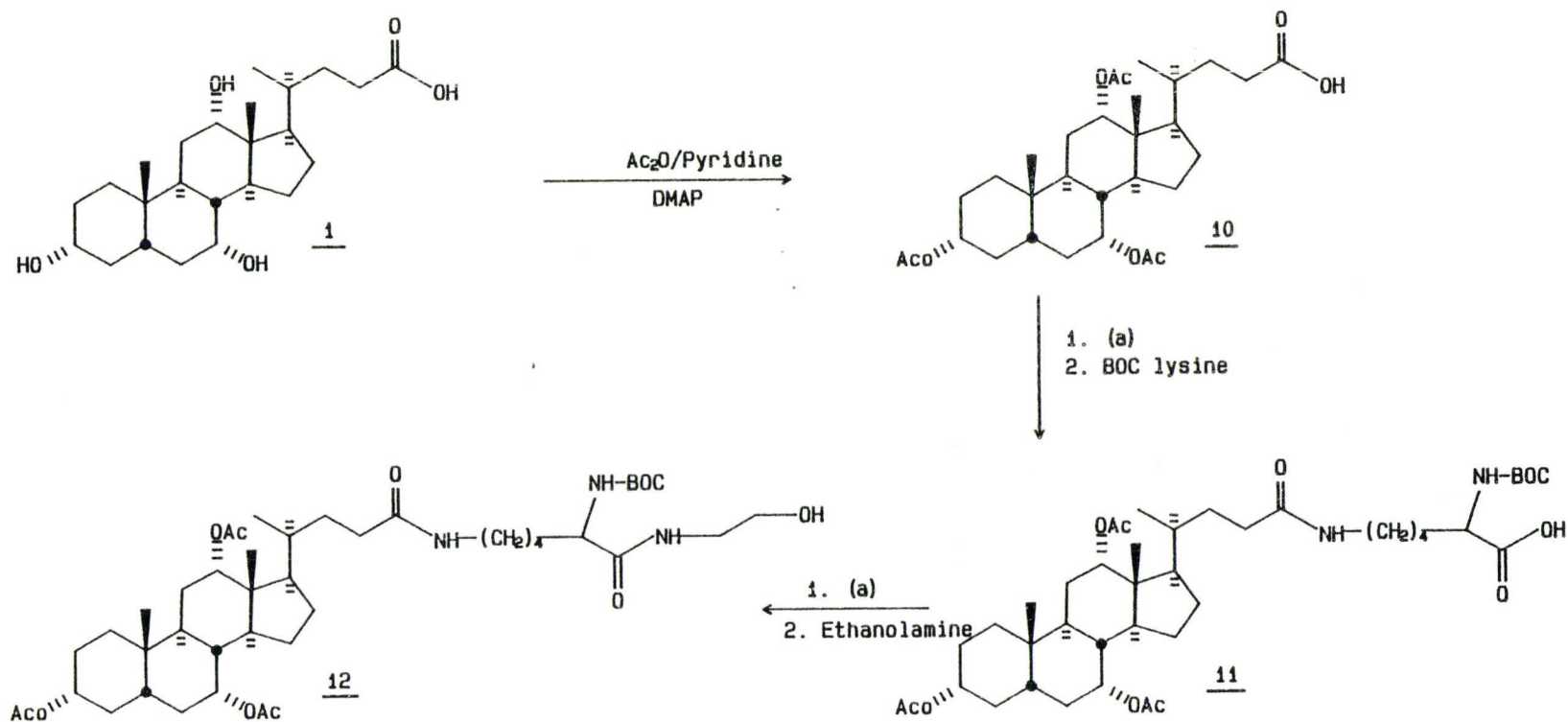


Fig 3.2 Mechanism for the mixed carbonic anhydride method for carboxyl group activation.

proton NMR spectrum at 1.4 ppm as well as the presence of the two broad NH resonances at 5.7 and 6.4 ppm which correspond to the amide and carbamate protons. Mass spectrometry showed a $M+1$ peak at 763. The same reaction conditions were used for the next reaction in which ethanolamine was attached to the new carboxyl group via an amide linkage. The proton NMR spectrum of 12 now showed three broad NH signals (5.3, 5.8 and 6.8 ppm) and two additional multiplets from the ethanolamine moiety at 3.5 and 3.7 ppm which were not present in compound 11. Mass spectrometry confirmed the structure by showing a $M+1$ peak at 807. See Scheme 3.3 for reaction sequence.

Scheme 3.3

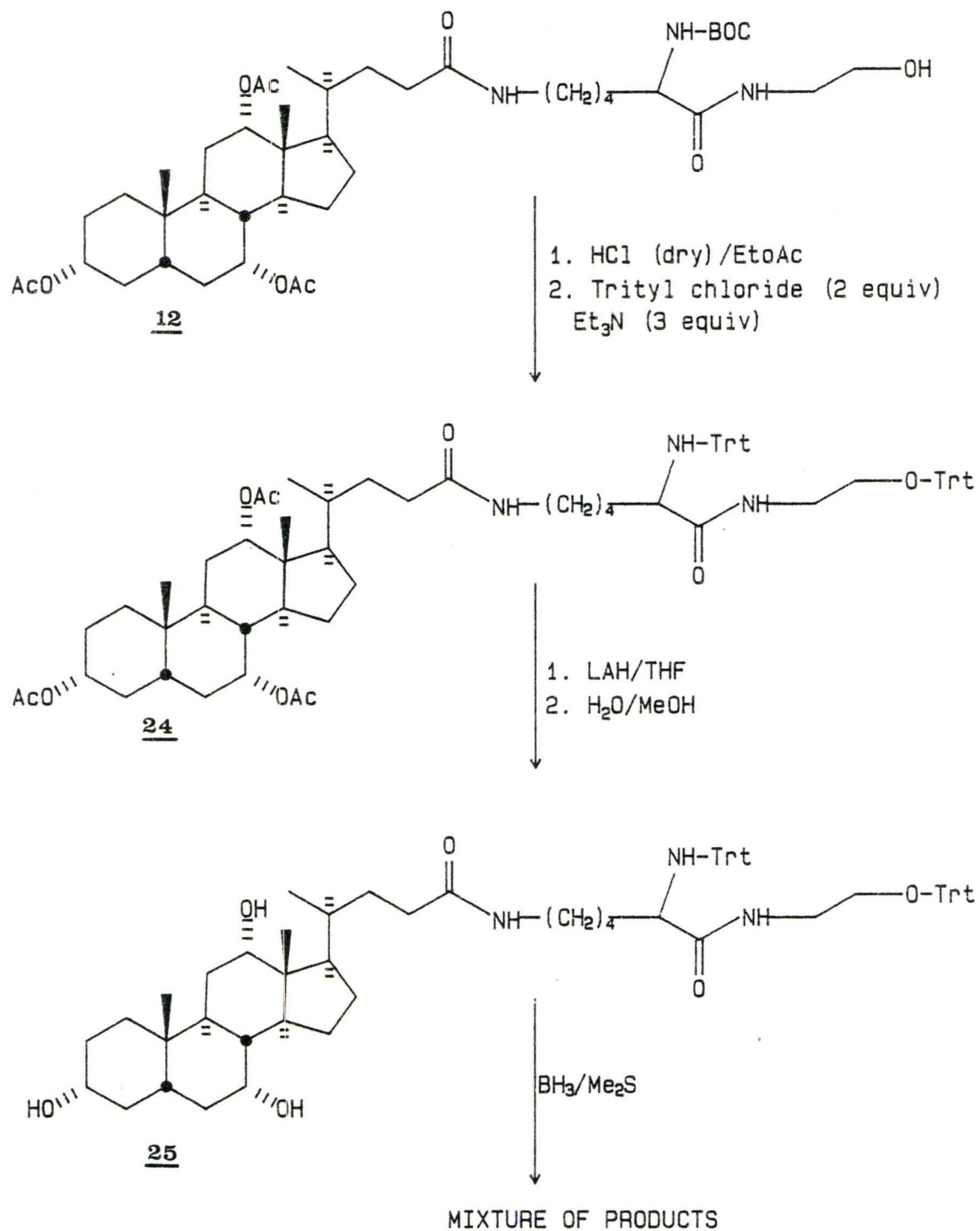


(a) = isobutyl chloroformate / triethyl amine (-20 C)

3.1.3 Trial Experiments

Continued experimentation on this synthesis (Scheme 3.3) was undertaken in an exploratory fashion. Removal of the BOC protecting group was attempted first and was achieved using dry HCl gas, but removal of the acetates however was not as successful. Hydrolysis of the acetates was first attempted using potassium carbonate in methanol and later sodium hydroxide in aqueous methanol with no success. The Cl₂ acetate is apparently quite hindered (Fieser and Fieser, 1959) and remains intact even under fairly rigorous hydrolysis conditions. Removal of the acetates was then attempted with lithium aluminum hydride (LAH). Previous experiments carried out by H. Hunter (Hunter, 1987) had shown that lithium aluminum hydride was not effective at reducing amides on long chain compounds with a free primary amine, unless the amine was previously protected. Coordination of the amine with LAH is expected to be the cause. Protection of the amine was therefore required. The BOC group was not suitable under reductive conditions and was therefore removed (dry HCl gas) and replaced with a trityl group (trityl chloride and triethyl amine) to produce 24. This was achieved using excess trityl chloride in an attempt to protect both the amine and the primary hydroxyl group. Preliminary NMR results looked promising as the trityl group signal of the product was apparent in the proton NMR spectrum (broad aromatic

Scheme 3.4



resonances at 7.1 ppm). The product was also apparent as a single UV sensitive spot on TLC. Reduction of the acetate esters was then attempted using LAH and proved reasonably successful. The proton NMR spectra of 25 showed that the three singlets at 2 ppm corresponding to the acetate methyl groups had disappeared and the proton signals on carbons 3, 7 and 12 had moved upfield by approximately 1 ppm (where they are normally located in cholic acid when the hydroxyls are not protected). Further reduction of the amides was then attempted on the crude product using a borane dimethyl sulphide complex. The reaction led to a number of reaction products as seen by TLC and did not look promising.

At this stage it was apparent that the synthesis was growing in length from what was originally expected. With convenience and practicability in mind, a more direct synthesis was preferable and other synthetic strategies were therefore explored.

3.2 SYNTHESIS OF CHOLIC ACID-EDTA

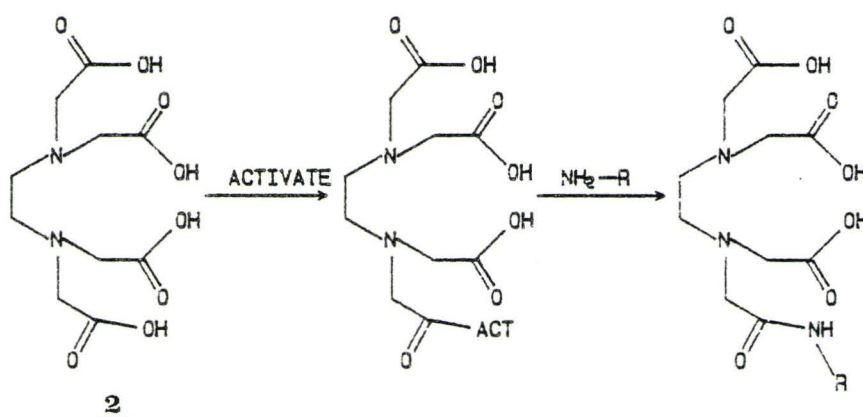
3.2.1 Synthetic Strategy

The synthetic strategy for the production of 7 was very simple. It was possible to use relatively few synthetic steps and inexpensive starting materials. EDTA itself was used as a starting material and by appropriate activation of one carboxyl group on EDTA, followed by reaction with a biological molecule possessing free hydroxyls, thiols or

primary amines, a biomolecule-chelate linkage would be obtained. This methodology could then be used as a general procedure for a vast number of biological molecules containing these functional groups.

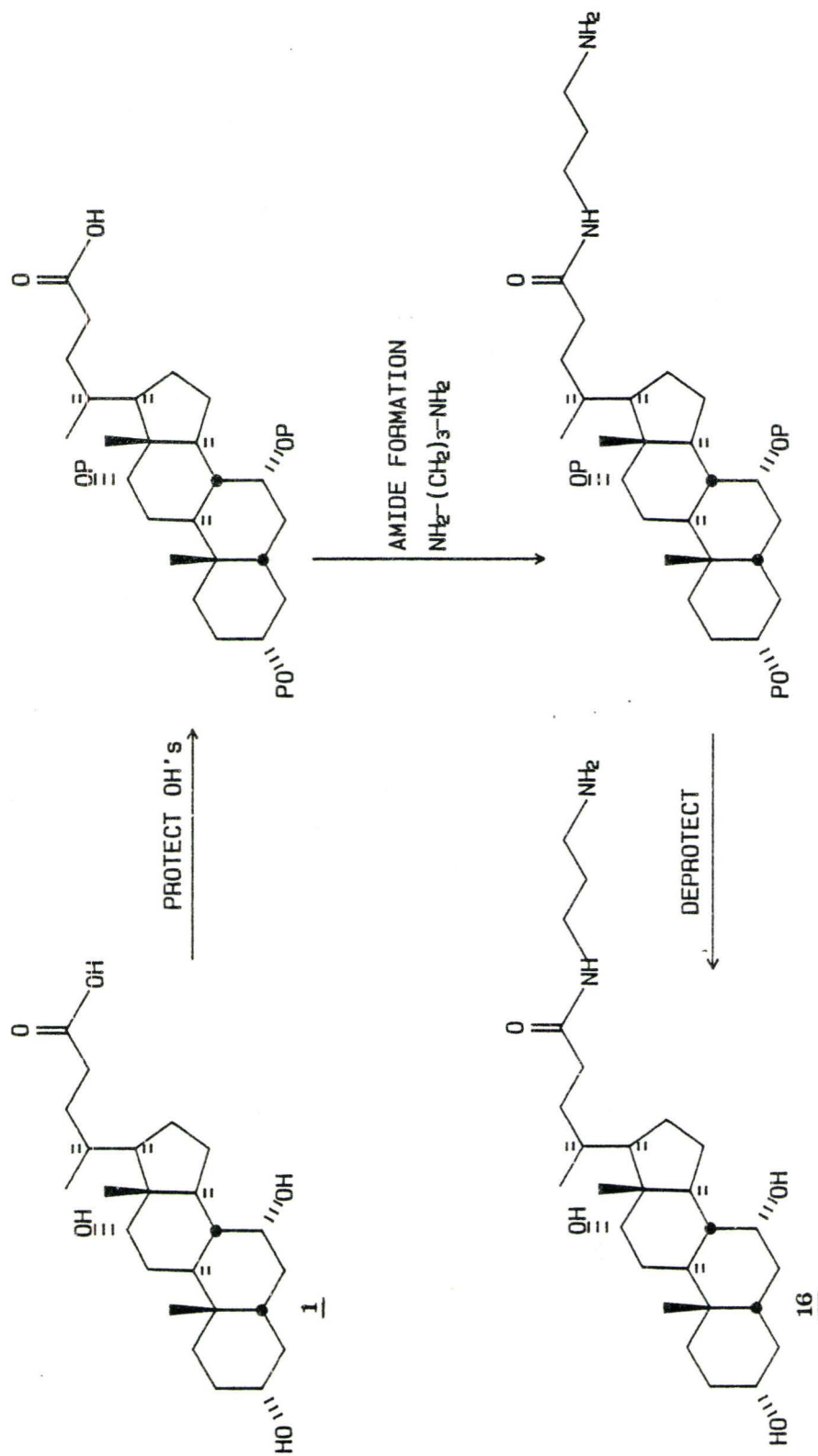
The formation of an amide linkage with EDTA was preferable to ester or thiol ester linkages due to its relatively higher stability towards hydrolysis at physiological pH's. To form the amide linkage to EDTA,

Scheme 3.5



cholic acid must first be converted to a primary amine. A diamine such as diaminopropane could be used as a spacer chain to link cholic acid to the chelating moiety. Formation of an amide with cholic acid would mimic the naturally occurring conjugate bile acid and would in turn leave the primary amino group at the other end of the diamine free for later reaction with EDTA. Since there are free hydroxyl groups on cholic acid which would interfere with amide formation using standard coupling reagents, they must first

Scheme 3.6



be protected. This sequence of manipulations is illustrated in Scheme 3.6.

Removal of the hydroxyl protecting groups followed by reaction of the amine with activated EDTA would produce the final product as illustrated earlier in Scheme 3.5.

3.2.2 Synthesis

Activation of a carboxyl group on EDTA was found to be unsatisfactory due to the insolubility of EDTA in all solvents except DMSO and DMF (only slightly soluble). Furthermore, for any activation procedures that could be carried out in these solvents, there existed the statistical problem of activating only one carboxyl group, or of separating mixtures of mono, di, tri, and tetra-activated EDTA. Di-activation could however be accomplished by the formation of the dianhydride (EDTADA)(13) by means of a heterogeneous reaction in acetic anhydride and pyridine (Geigy, 1968). The dianhydride was formed as a pure crystalline product which was stable when stored at room temperature in a desiccator. The infrared (IR) spectrum of 13 showed bands at 1770 cm^{-1} corresponding to the anhydride carbonyls, and proton NMR spectrum showed two singlets (2.6 and 3.7 ppm) in a ratio of 1:2 (see Fig 3.3). Di-activation in this manner reduces the statistical problem to reaction at one of two sites rather than one of four,

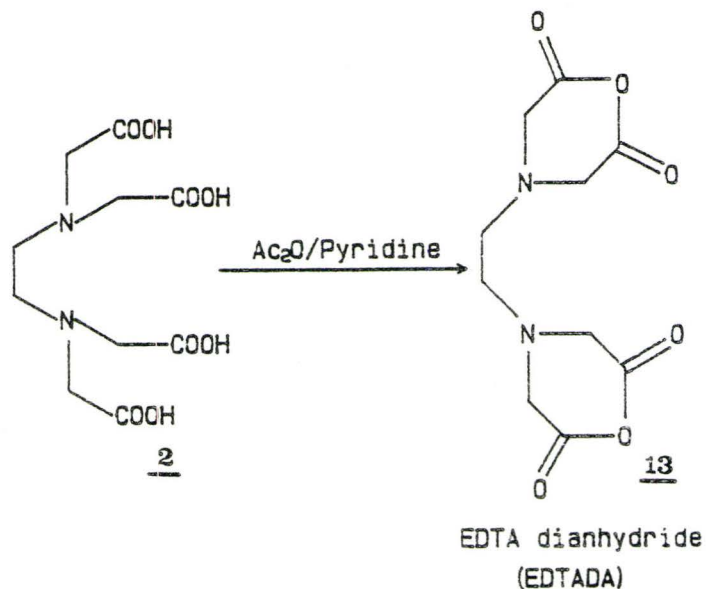


Fig 3.3 Reaction of EDTA with acetic anhydride in pyridine to form the dianhydride.

and therefore reduces the number of possible reaction products.

Conversion of cholic acid to an amine was attempted as outlined earlier by reaction with diaminopropane. Protection of the hydroxyl groups was required first. Previous experiments showed this could be done effectively by acetylating them but the acetates were difficult to hydrolyse and must be removed reductively with lithium aluminum hydride. Formylation of the hydroxyls was then attempted and was found to give a crystalline product (19) in high yield. The reaction was performed by treating cholic acid with formic acid and acetic anhydride with perchloric acid as a catalyst. The reaction mechanism is shown in Fig 3.4). The proton NMR spectrum for compound 19 showed the

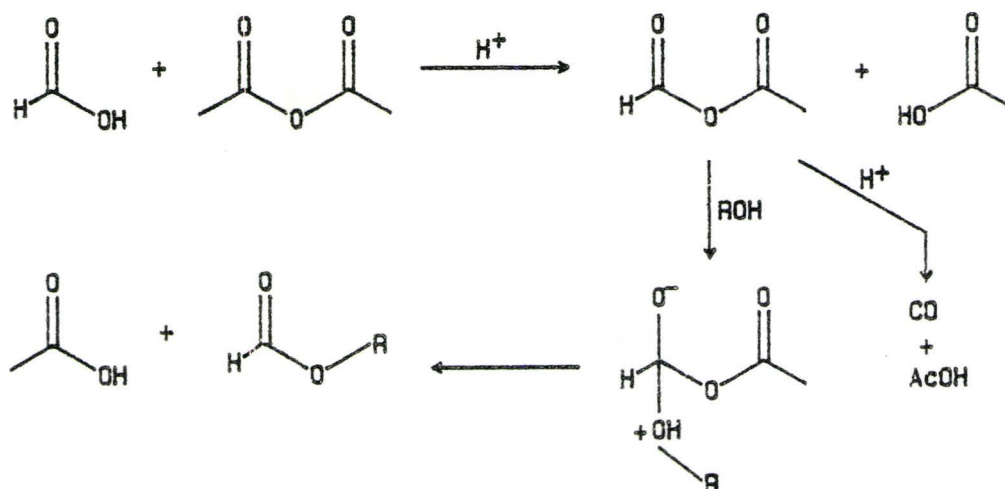
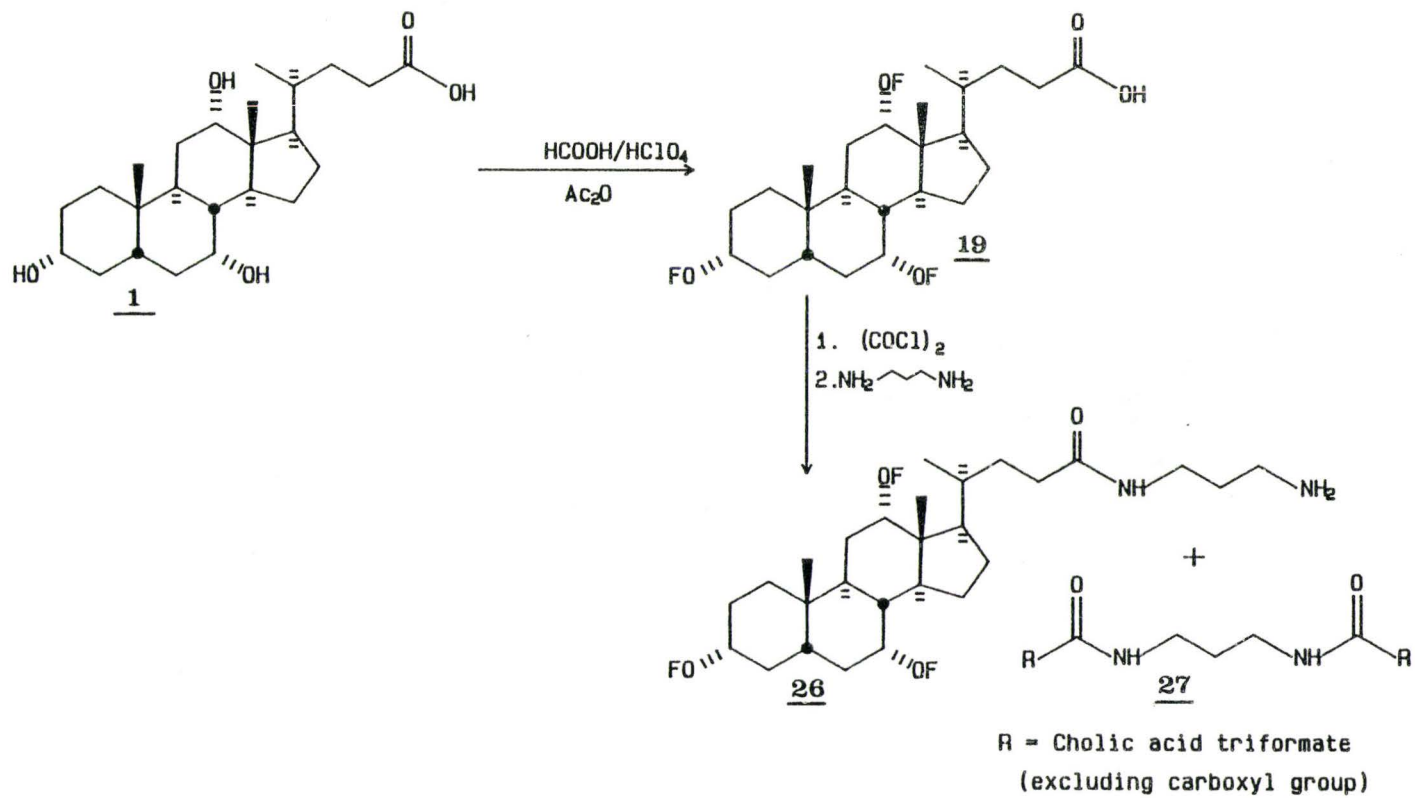


Fig 3.4 Reaction mechanism for the formylation of the three hydroxyl groups on cholic acid

appearance of three singlets at approximately 8 ppm corresponding to the three formyl protons, and showed the same downfield shift of the protons attached to the formylated carbons as did the triacetate (approx. 4.7 to 5.2 ppm). Mass spectrometry showed a M-1 ion at 491. Preliminary experiments showed the formates (including the hindered C12-formate) could be removed easily by base hydrolysis and therefore were much more suitable for protecting groups in this case.

The amide bond was formed by conversion of 19 to an acid chloride with the use of oxalyl chloride, followed by reaction with excess diaminopropane to produce 26. The reaction was performed by dropwise addition of the acid chloride to a solution of excess diaminopropane in tetrahydrofuran (THF) at $-78\text{ }^\circ\text{C}$ (Scheme 3.7). Even at great dilution and low temperature, significant amounts of

Scheme 3.7



the diamide (27) were formed (see Scheme 3.7). Separation of the products was difficult as the desired monoamine product (26) could not be extracted into an acidic aqueous layer due to its large size and hydrophobic character. Reaction at both ends of the diamine was attributed to the formation of micelles by cholic acid triformate in solution (Fieser and Fieser, 1959). In doing this, the steroid molecules are aggregated in solution and are spatially arranged for "direaction" (see Fig 3.5). Yields were therefore

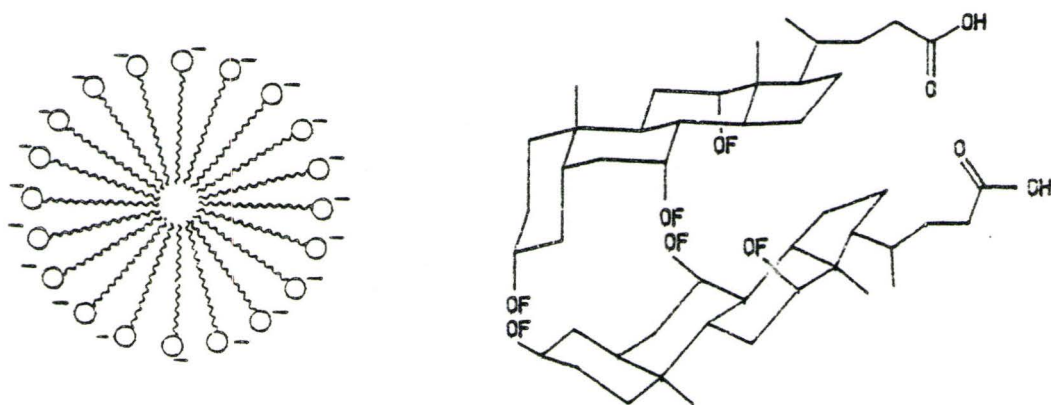
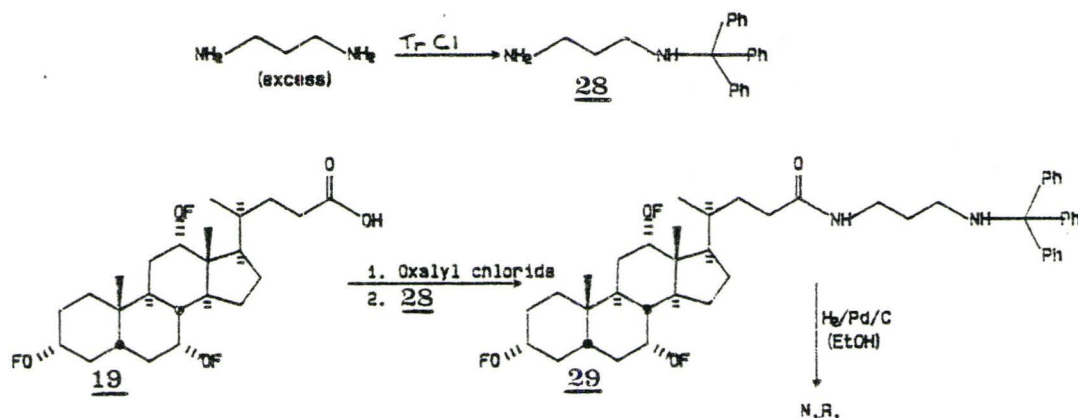


Fig 3.5 Diagram depicting micelles and cholic acid dimers in solution.

unsatisfactory. Mono-protection of the propane diamine was a possible solution to this and could be accomplished by the reaction of trityl chloride with a large excess of the diamine at low temperatures. Separation of the product (28)

from the unreacted diamine was trivial as the diamine was quite water soluble and could even be extracted into a slightly basic aqueous layer (to ensure that 28 was not protonated) while 28 remained in the organic layer. Attachment of the mono trityl diamine to cholic acid via the acid chloride proceeded smoothly to give the amino protected cholic acid derivative (29), but removal of the trityl group by hydrogenation was later found to be unsuccessful.

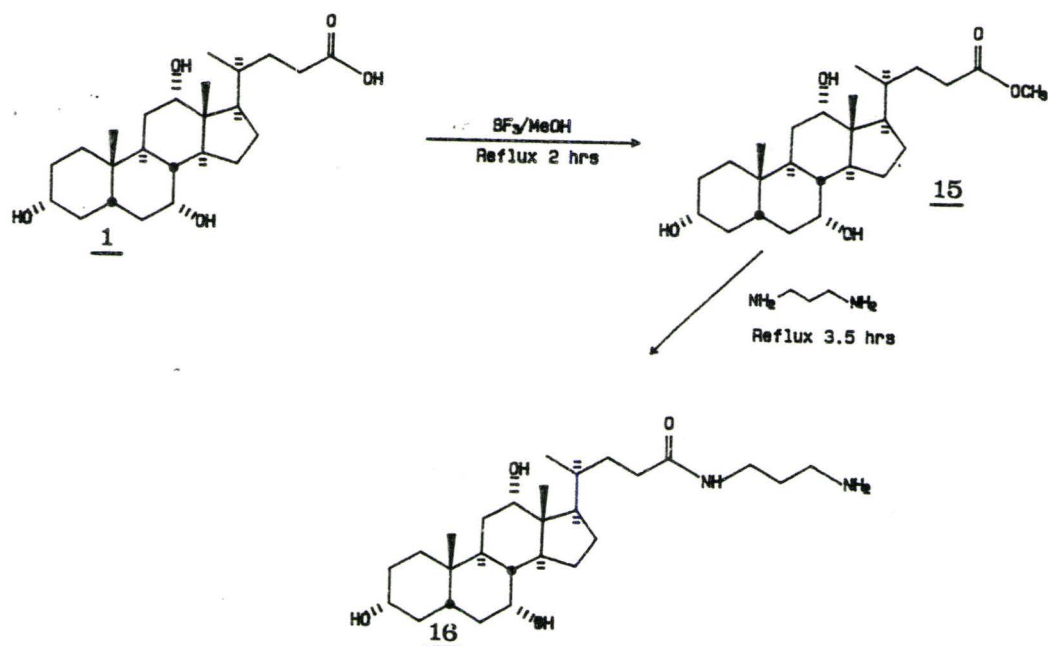
Scheme 3.8



At this point a slightly different approach was sought. Could a much less reactive activating group be used that would i) react with the diamine slowly to avoid the diamide formation and ii) be sufficiently unreactive so the hydroxyl groups need not be protected? This was in fact achieved through the use of cholic acid methyl ester (15). Reaction of 15 in neat diaminopropane virtually removed all

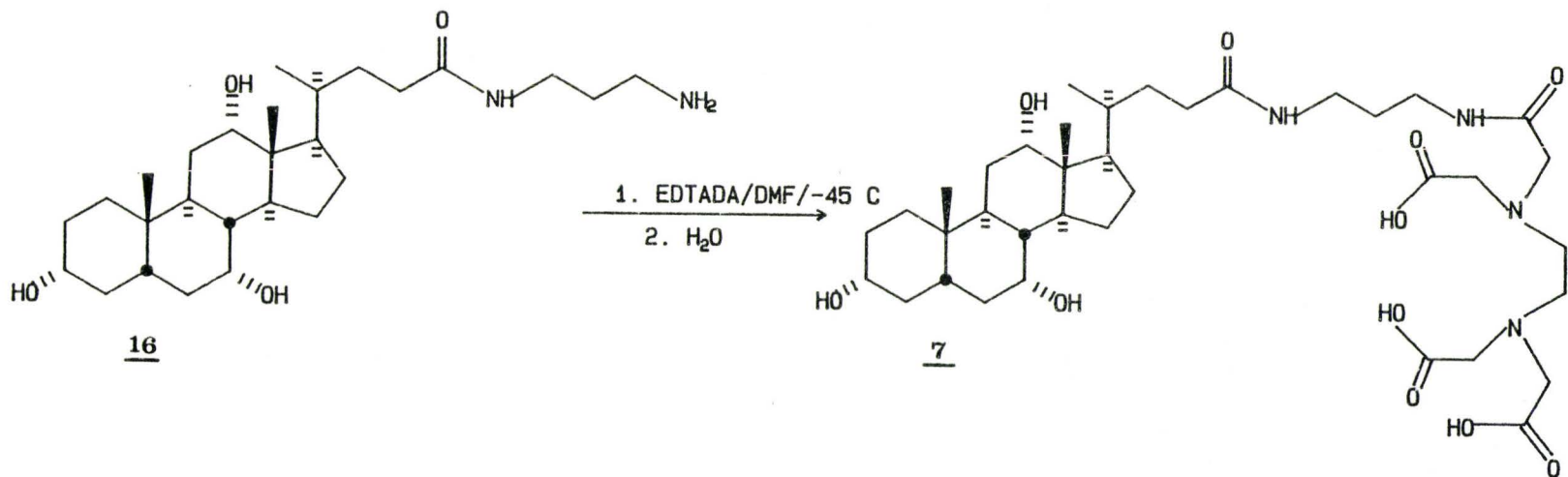
possibility of dimer formation and produced the amide in good yield without the need for hydroxyl protection. Compound 16 showed a single spot on TLC and was ninhydrin sensitive which is indicative of primary amines. The proton NMR spectrum showed the presence of two triplets at 2.6 and 3.2 ppm corresponding to two nonequivalent methylenes next to the amine and amide nitrogens. Mass spectrometry showed a M+1 peak at 465.

Scheme 3.9

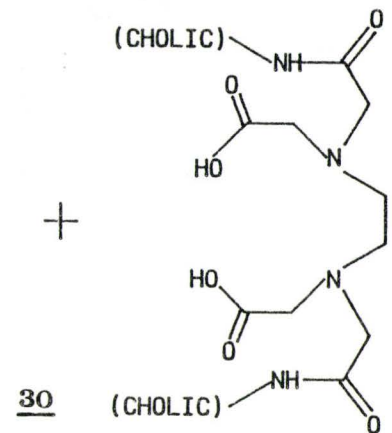


Coupling of the steroidal amine (16) with the EDTA dianhydride was attempted several times by the addition of 16 in dimethylformamide (DMF) to a solution of EDTA

Fig 3.6 Reaction and table depicting the formation of two products from the reaction of 16 and 13.



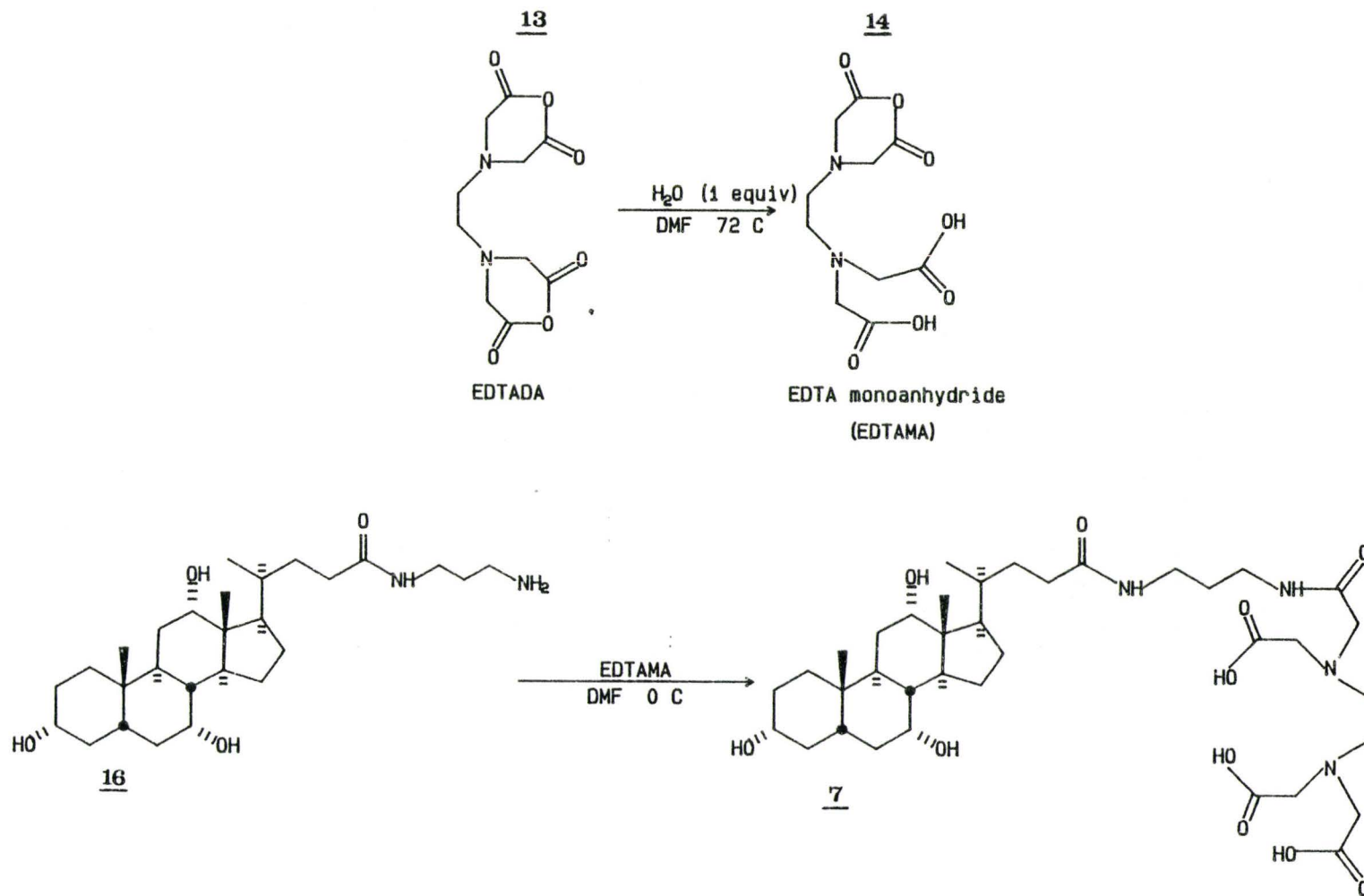
% Compound 30	Temperature (°C)
70	20
50	0
30-60	-65



dianhydride in DMF at various temperatures. As was expected, the hydroxyl groups were far less reactive than the amine and did not react with the anhydride sites under these conditions. Production of "dicholic-EDTA" (30) where the amine had reacted at both ends of the dianhydride was a very significant reaction product (often the main product as evidenced by TLC analysis). The extent to which this side reaction occurred was dependent on temperature (see table in Fig 3.6) as well as on traces of water in the DMF. Separation of the desired product from the di-reacted side product (30) was very difficult as the two compounds had similar solubility and chromatographic properties.

In order to solve the problem of dimer formation, the dianhydride was partially hydrolyzed to form the monoanhydride (EDTAMA) (14) (Takeshita et al, 1982). This was performed by the addition of one equivalent of water to a saturated solution of EDTA dianhydride in DMF. Under these conditions one would expect to get a statistical mixture (1:2:1) of dianhydride, monoanhydride and tetra-acid, respectively. EDTA monoanhydride, however, is much less soluble than the other two compounds and precipitates out of solution immediately driving the reaction to the formation of one product only. The proton NMR spectrum of the mono-anhydride differed greatly from that of the dianhydride. The methylenes on the ethylene diamine backbone of 14 were no longer equivalent, but instead were

Fig 3.7 Formation of EDTA monoanhydride and its use in obtaining pure Cholic acid-EDTA

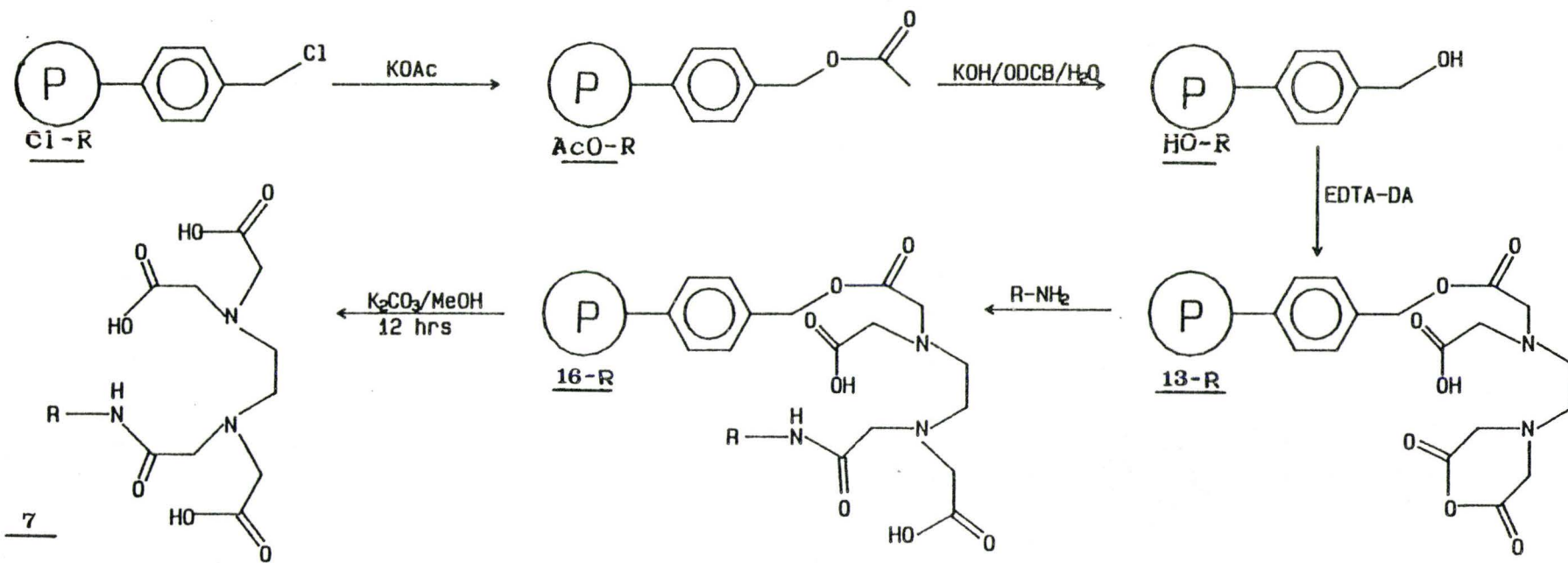


coupled to each other and appeared as two triplets at 2.6 and 2.8 ppm. Two other singlets were present which corresponded to the methylenes next to the anhydride (3.7 ppm) and carboxylic acids (3.4 ppm). The IR spectrum showed the presence of both anhydride (1770 cm^{-1}) and carboxylic acid (1640 cm^{-1}) absorptions. Trial experiments using the monoanhydride and refluxing methanol showed the monomethyl ester as the only reaction product. Reaction of the monoanhydride with 16 also gave only one product (7) and in excellent yield. Compound 7 showed a strong M+1 peak in the mass spectrum at m/z 739. Signals corresponding to the chelating portion of 7 were apparent in the proton NMR spectrum as overlapping multiplets at 3.0 to 3.4 ppm and five peaks corresponding to the carbonyl carbons were present between 174 and 181 ppm in the C-13 NMR spectrum.

A solid phase synthetic approach was undertaken concurrently with less satisfactory results. Synthesis of the resin was performed using literature methods (Stewart and Young, 1969) and was monitored by IR spectroscopy. The reaction scheme is shown in Fig 3.10. All reactions on the resin were performed using excess reactants since removal of the excess was easily achieved by filtration.

The chloromethylated divinylbenzene polystyrene copolymer (C1-R) was obtained as a starting material. For use as a solid phase support in this synthesis it was first converted to the hydroxyl form (HO-R). This was accomplished

Scheme 3.10



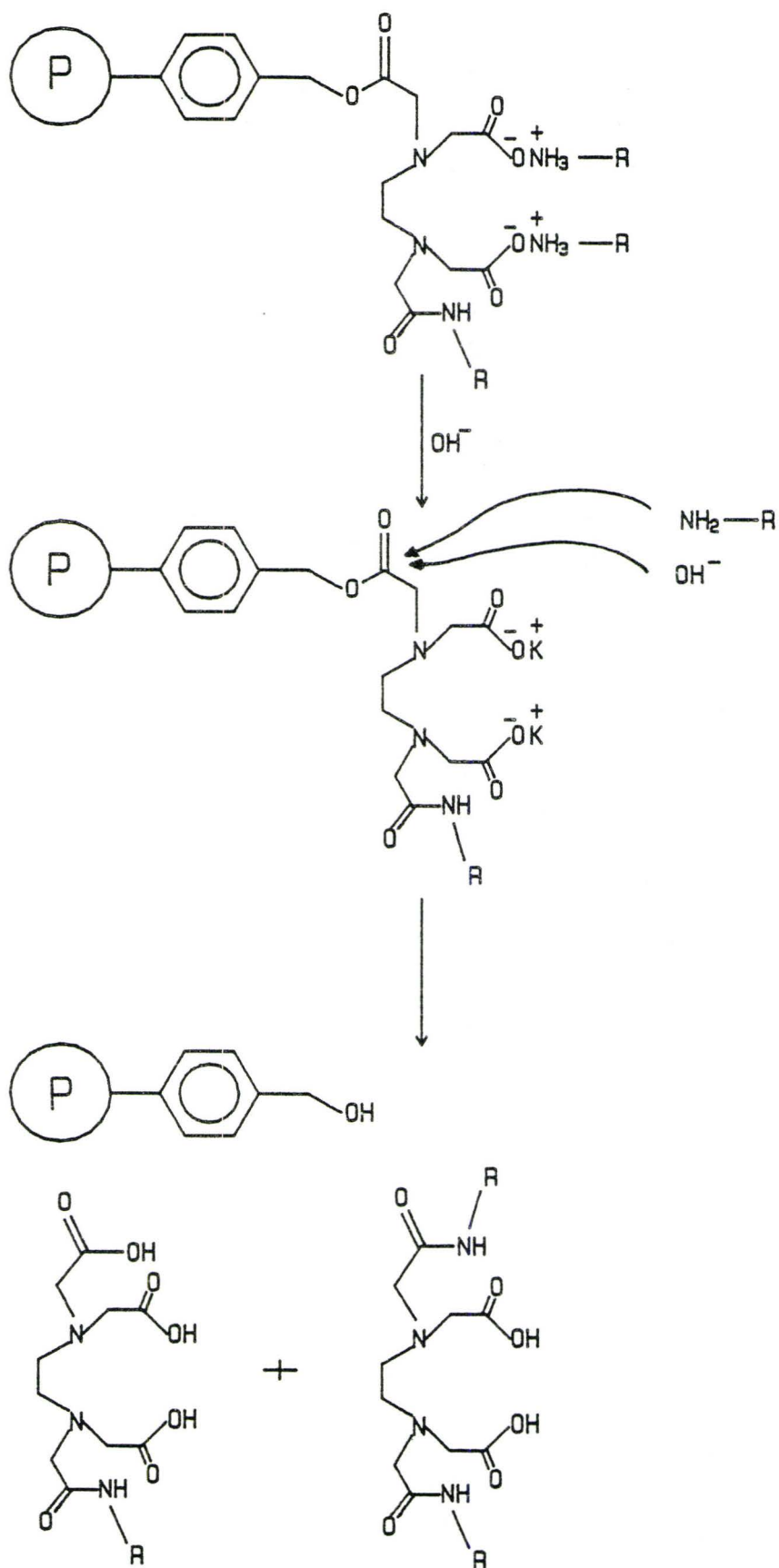
by making the acetoxy resin (AcO-R) by the substitution of chloride for acetate followed by ester hydrolysis. Ester absorptions (1735 cm^{-1}) in the IR were present for the acetylated resin while OH absorptions could be seen at 3440 and 3560 cm^{-1} for the hydroxyl case.

Attachment of the dianhydride to the hydroxy resin to produce 13-R was the first step in the synthesis. This was done by heating a concentrated solution of 13 with the hydroxy resin. Attachment was confirmed by the presence of anhydride bands at 1820 cm^{-1} in the IR spectrum. No quantitative information was acquired to determine the extent of the reaction (This could have been done by hydrolysis of the anhydrides and titration of the carboxylic acid groups). Compound 16 was then attached by mixing it with 13-R at cool temperatures. The IR spectrum of 16-R showed the disappearance of anhydride bands and the appearance of amide and hydroxyl bands. The final step in the synthesis was to hydrolyze the ester linkage to the resin and recover the final chelate free from dimer (30), as was the problem when using 13 in a solution phase approach. Upon hydrolysis two products were recovered: compounds 7 and 30. How could there be "dicholic-EDTA" formation if one end of the dianhydride had already reacted with the resin? There are two possibilities for this occurrence i) the hydroxy resin has a strong physical adsorption for 13 so it could be bound to the resin without having reacted with it,

and ii) 16 is reacting at the ester linkage where 13 was attached to the resin as well as at the anhydride sites. Solid phase supports usually have little or no adsorption properties so ii) was suspected as the likely cause. This in fact seemed quite likely the case since after reaction of 16 with the anhydride site there are two free carboxyl groups, and the resin behaves as a cation exchange resin and binds excess 16 ionically. When the resin is exposed to basic conditions, as is the case for ester hydrolysis, the amine is freed from the resin and is available for nucleophilic attack at the ester linkage. Rates for base hydrolysis and nucleophilic attack at the ester site in this case appear to be comparable since two products are formed. To alleviate this problem the resin was washed with dilute acid to remove any unreacted 16 before ester hydrolysis. Hydrolysis in this instance gave 7 (identical to that produced by monoanhydride method) as the only product. The yield however was poor (21%) and dibonding of the dianhydride to the resin was the suspected cause. The use of a resin with a different cross linkage or smaller concentrations of functional groups per mass of resin may have improved yields significantly. This however was not attempted since the monoanhydride approach had proven superior.

3.2.3 Other Trial Experiments Towards Cholic Acid-EDTA

As possible alternatives to the anhydride activation



Scheme 3.11

procedure, two other methods were concurrently investigated. One approach, which had precedent in the literature (Hertzberg and Dervan, 1984), was to react the amine in DMF with an excess of EDTA with no prior carboxyl activation. Because reaction rates of carboxylic acids with amines are slow, diamide formation (from micelles in solution) should be negligible if a large excess of EDTA was used. The experiment was performed but EDTA has very similar solubility properties as 1 and separation of the product by crystallization could not be achieved. Chromatography on silica was also not a viable route because EDTA and 1 are insoluble in almost all organic solvents. Both compounds, however have varying solubilities in low molecular weight alcohols and aqueous buffers and therefore reverse phase chromatography may have been successful but was not attempted.

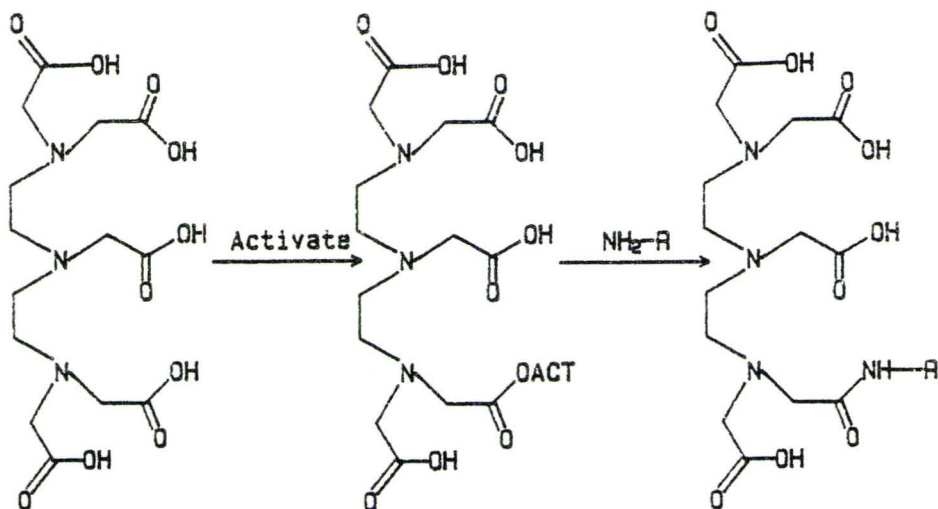
Another possibility was the use of EDTA tetramethyl ester (31). Again reaction rates would be slow enough to reduce "di-reaction" if a large enough excess of EDTA tetramethyl ester was used. Trial experiments were performed using octylamine and EDTA tetramethyl ester at 80°C and 155°C for varying times. Reaction was slow and unidentified side products were apparent from TLC and their rates of formation exceeded the rate of product formation. Both of these routes were unsatisfactory and were therefore abandoned.

3.3 SYNTHESIS OF CHOLIC ACID-DTPA

3.3.1 Synthetic Strategy

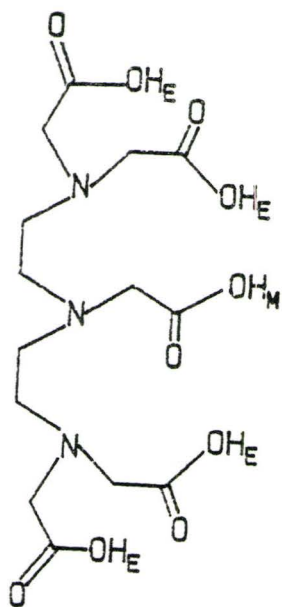
The strategy used for the production of cholic acid-DTPA was the same as that used in the production of cholic acid-EDTA. In this case the chelating moiety (DTPA) was used as a starting material. Activation of one carboxyl group followed by reaction with a molecule possessing a free amino group would lead to a stable amide linkage. Manipulation of cholic acid to introduce a primary amino group was discussed previously and was appropriate in the DTPA case.

Scheme 3.12



Since the synthetic strategy in this case is conceptually the same as that used in cholic acid-EDTA production, and incorporation of the chelating moiety is the last step, EDTA and DTPA could be interchanged to produce biological chelates with very different chelating properties. The production of chelates which have the same biological activity, but different chelating properties would be very beneficial for cases where different metals are to be chelated for use in different spectroscopic studies.

In the case of DTPA however, there is one problem with the existing strategy. The carboxyl groups are not all chemically equivalent. The four terminal carboxyl groups are the same but the central carboxyl group is different. Therefore, along with the problem of achieving mono-reaction



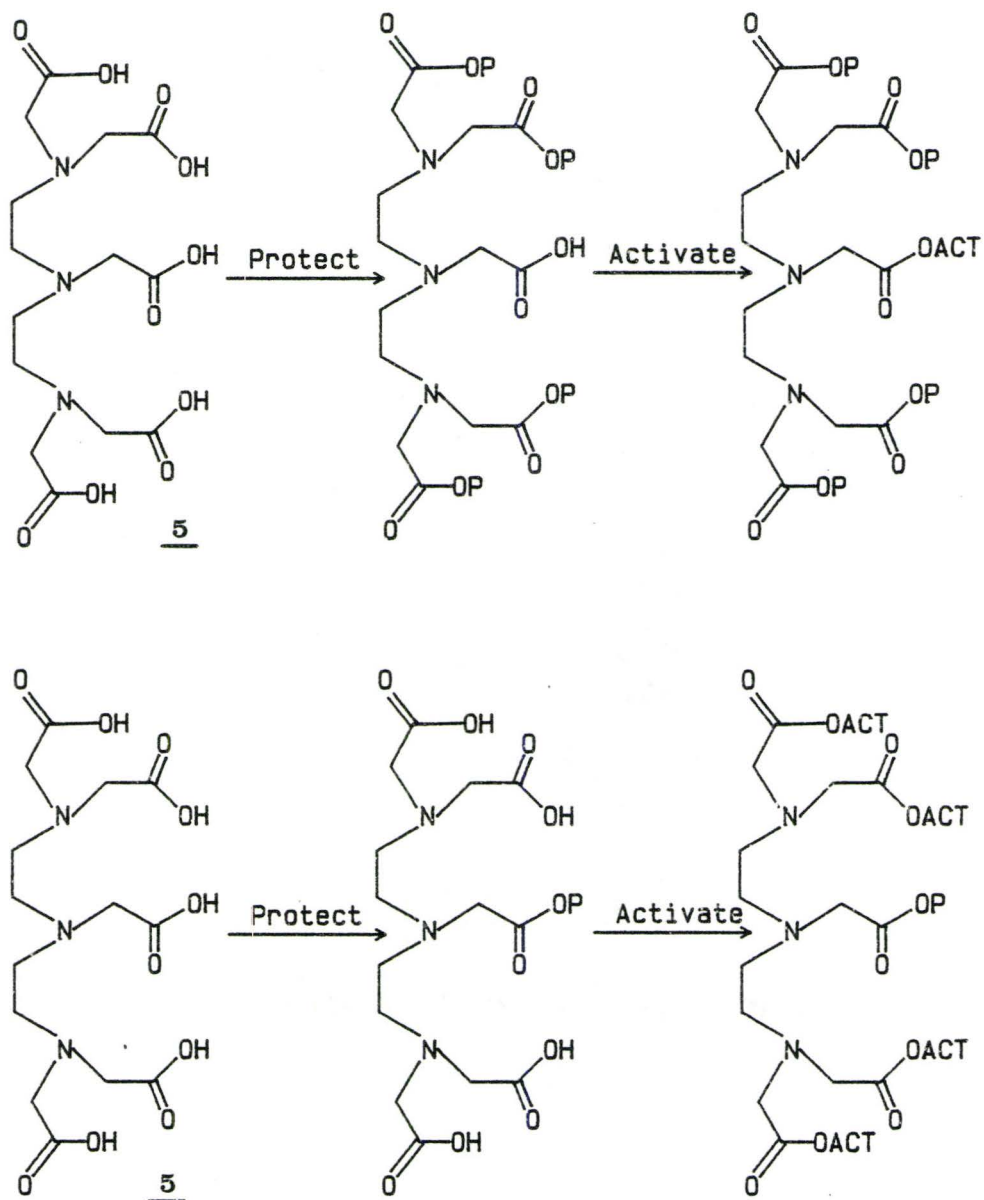
at one carboxyl site, there exists the problem of differentiating between the chemically different carboxyl groups. If differentiation could be achieved, then two DTPA bio-chelates could be produced, each with slightly different chelating properties. Therefore, selective procedures to distinguish between terminal

carboxyl groups and the central carboxyl group were considered and are depicted in Fig 3.8.

3.3.2 Synthesis

Mono activation of a carboxyl group on DTPA was difficult to achieve for the reasons already stated for EDTA. Furthermore, non-selective mono activation of the penta-acid would lead to a mixture (4:1) of end carboxyl activation, and central carboxyl activation respectively, and therefore lead to a mixture of products. Carboxyl group activation by the dianhydride approach was therefore undertaken as it clearly distinguishes end carboxyls from the central one by their ability to form six membered rings. The central carboxyl group does not react as it can not form a cyclic anhydride and mixed acyclic anhydrides are thermally unstable except at very low temperatures (March,1985). DTPA dianhydride (DTPADA)(17) was produced by reaction of DTPA with acetic anhydride in pyridine (Geigy,1968). A mass spectrum of compound 17 could not be obtained using electron impact, chemical ionization or fast atom bombardment techniques. The proton NMR spectrum showed two singlets at 3.3 and 3.7 ppm (ratio 1:4) for the methylenes next to the carboxyl group and the anhydrides, respectively, and two triplets at 2.6 and 2.7 ppm (ratio 1:1) for the methylenes on the diethylene triamine backbone. Partial hydrolysis of the dianhydride to the monoanhydride

Fig 3.8 Distinguishing end carboxyls from the central carboxyl



(32) was attempted several times by the addition of one equivalent of water in various solvent systems

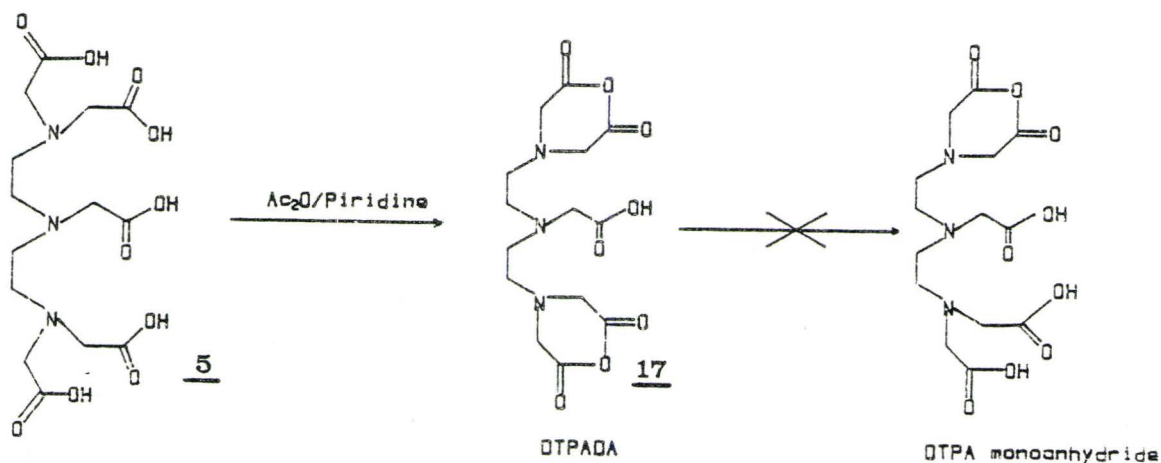
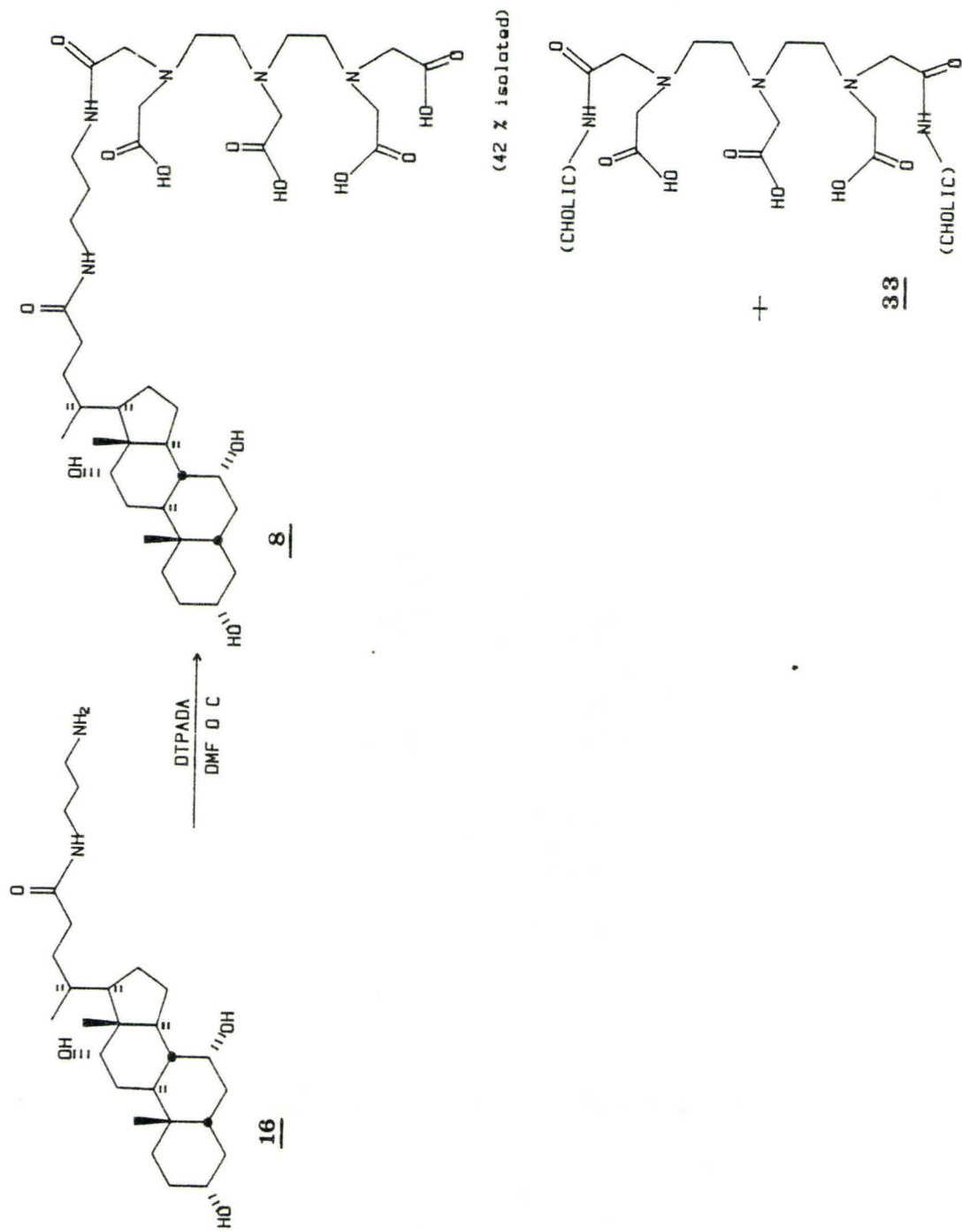


Fig 3.9 Reaction of DTPA with acetic anhydride in pyridine to give the dianhydride. Partial hydrolysis to the monoanhydride was not possible.

(DMF and DMF / dioxane combinations) with no success. Apparently the monoanhydride is more soluble than the dianhydride and the monoanhydride did not precipitate from solution. The proton NMR spectrum showed reaction mixtures to contain statistical mixtures of monoanhydride, dianhydride and penta-acid.

Reaction of the dianhydride with the biological amine (16) led to formation of the desired product 7, but was contaminated with significant amounts of the "dicholic-DTPA" product (33). Attempts were made to reduce the amounts of 33 in the product by using a large excess of DTPA

Scheme 3.10

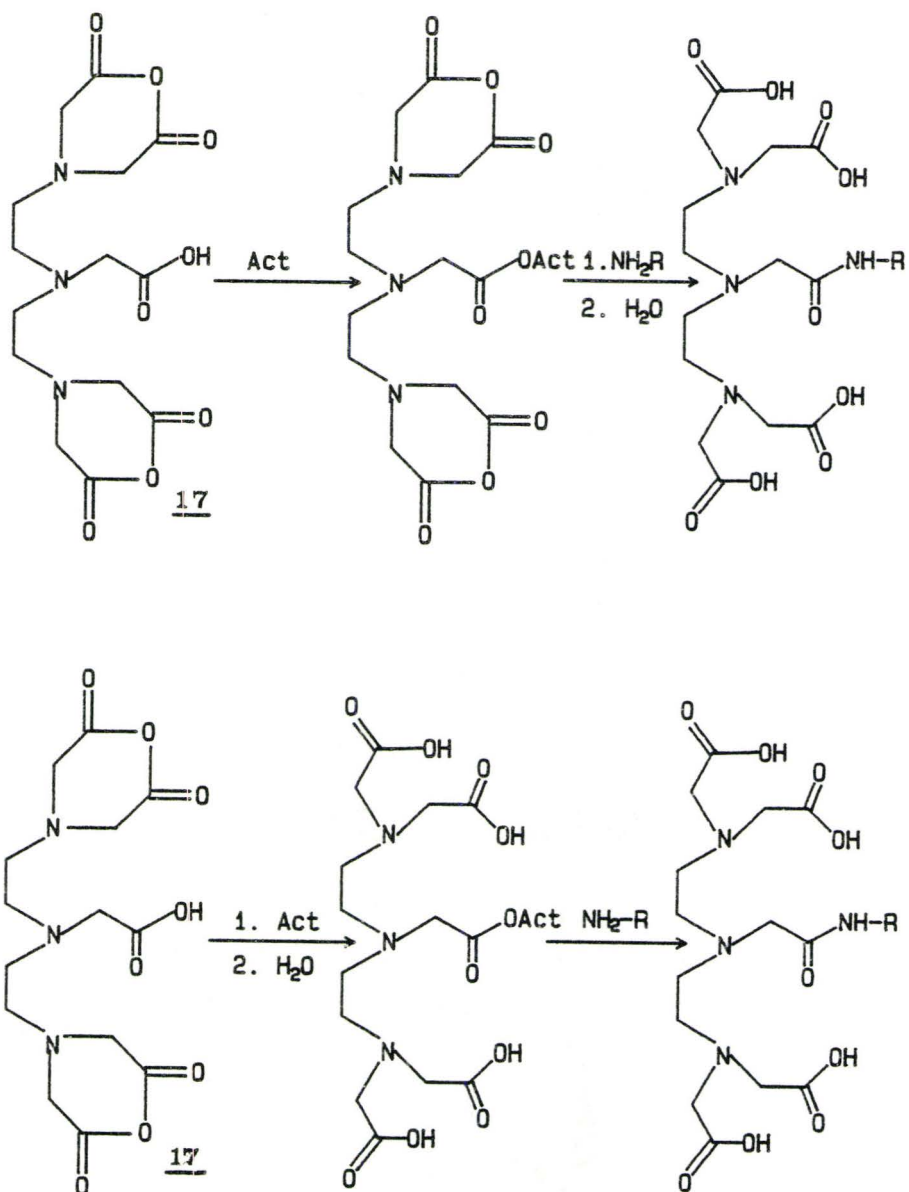


dianhydride, dilute solutions and very low temperatures. Significant amounts of 33 were formed in all cases and were separated from the desired mono-reacted product by chromatography. Like its EDTA analogue 8 showed an intense M+1 peak (at m/z 840) in its mass spectrum. The proton NMR spectrum showed overlapping multiplets at 3.1 to 3.4 ppm corresponding to signals from the chelating portion of the molecule which contains nine methylenes of very similar chemical shift. Solid phase synthesis was not attempted in this case since yields were very low in the analogous synthesis of cholic acid-EDTA.

3.3.3 Other Trial Experiments Towards Cholic Acid-DTPA

A second approach was undertaken to link DTPA to cholic acid through functionalization at the central carboxylic acid group. The most direct method seemed to be the dianhydride approach. This approach would utilize the major chemical difference between the end carboxyls and the middle carboxyl, namely the ability to form stable six membered rings. A drawback to this method is that the protecting groups (the anhydrides) are extremely reactive towards the steroidal amine (16). There are two ways to circumvent the problem i) introduce a "super reactive" activating group at the central carboxyl group so that 16 reacts there much faster than at the anhydride sites, or ii) introduce a "mildly reactive" activating group at the

Fig 3.11 Selective activation strategies for end and central carboxyl groups



central carboxyl group, hydrolyse the anhydrides with water, and then introduce 16.

Both approaches were examined, and both were unsuccessful. The "super reactive" activation procedure was undertaken first. Attempts were made to prepare the acid chloride and the mixed sulphonic anhydride (Chandrasekaran and Turner, 1982) (see Fig 3.13) using the reagents oxalyl chloride and methane sulphonyl chloride, respectively. Both attempts resulted in black tarlike substances depositing on the bottom of the flask and were therefore abandoned. The acid imidazolide (see Fig 3.12) was prepared by the use of carbonyl diimidazole as an activating reagent (reaction

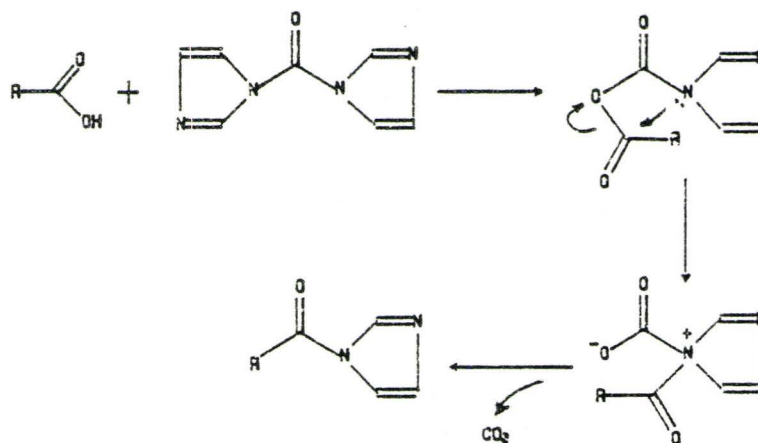


Fig 3.12 Mechanism of acid imidazolide formation using carbonyl diimidazole.

confirmed by slow evolution of carbon dioxide gas); its reactivity however was apparently comparable to the cyclic anhydrides because addition of an amine (butyl amine for

trial studies) gave a mixture of products as evidenced by TLC. Reaction was suspected to have occurred at both the anhydride sites and at the acid imidazolide.

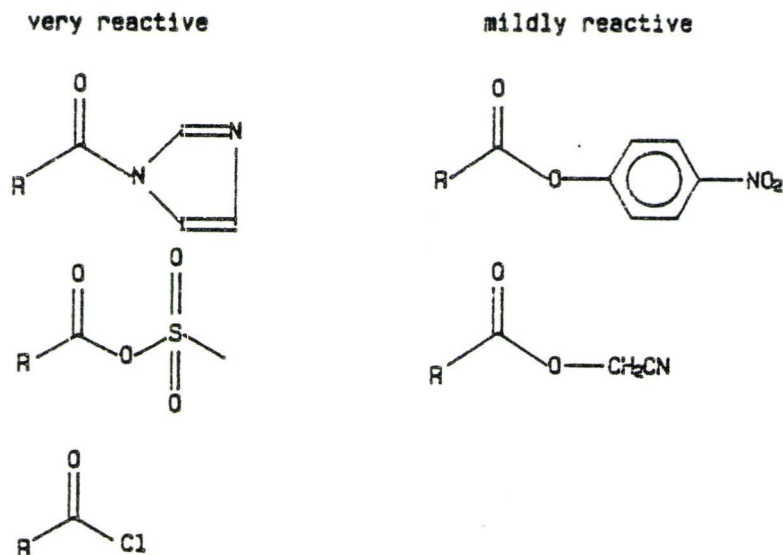


Fig 3.13 Activated carboxylic acids

The "mildly reactive" activation procedure was examined next. In one experiment chloroacetonitrile was used in an attempt to form the cyanomethyl ester, and in another experiment dicyclohexyl carbodiimide (DCC) and para-nitrophenol were used in an attempt to form the p-nitrophenyl ester. Following formation of the active esters the cyclic anhydride groups were hydrolysed by the addition of water and an amine was added to the hydrolysate

(butyl amine was used here for trial studies). The amount of amide formation was measured by proton NMR. Cyanomethyl ester formation was found to proceed in low yield (20-30%). Conditions for this reaction however were not optimized, and further work could possibly improve the yield to an acceptable level. Paranitrophenyl ester formation did not proceed at all. Possible steric interactions between the cyclic anhydride groups of the dianhydride and the cyclohexyl groups on DCC might account for this failure to react.

3.4 CHOLIC ACID-DHPTA

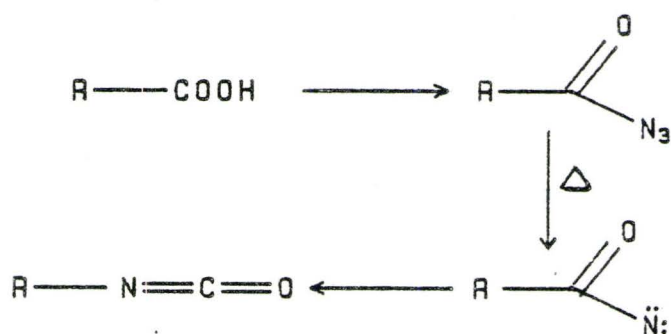
3.4.1 Synthetic Strategy

As in the synthesis of 7 and 8, the chelating moiety (in this case DHPTA) was to be used as a starting material. However, DHPTA was to be functionalized at the secondary hydroxyl group rather than at a carboxyl group as in the case of 7 and 8. Incorporating DHPTA in this manner eliminates the problem of multifunctionality where reaction is to occur at a carboxyl site and also does little to alter the chelating characteristics of the DHPTA derivative since the hydroxyl group is not directly involved in chelation. Because the hydroxyl group is sterically hindered, coupling of the chelating moiety requires a biological molecule which is highly reactive towards alcohols. Isocyanates are highly reactive towards alcohols and form carbamates which show

relatively high resistance to hydrolysis.

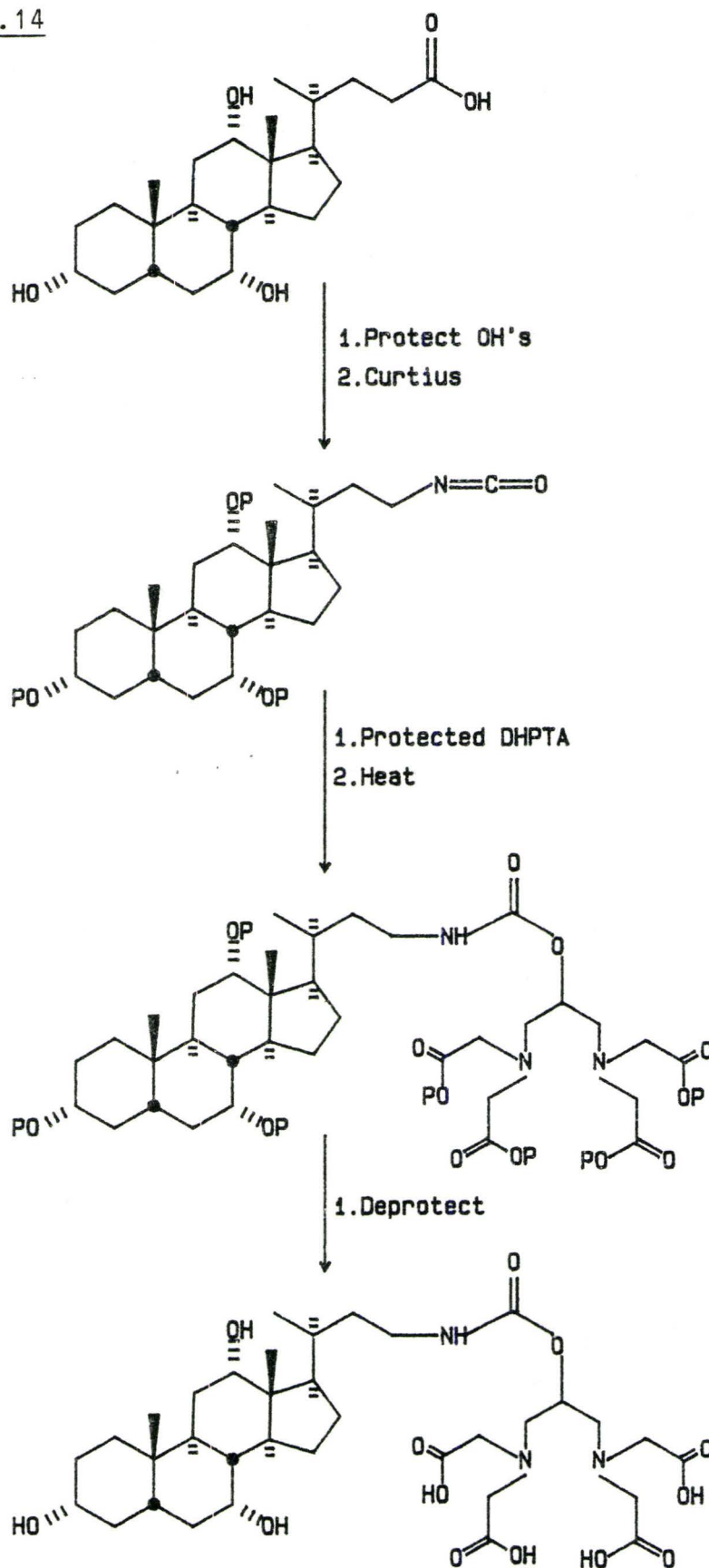
In order to introduce an isocyanate group onto cholic acid, the hydroxyl groups must be protected. An appropriate method for isocyanate formation was to make an acid azide and subject it to the Curtius rearrangement (see below). This could be carried out on the protected cholic

Scheme 3.13



acid itself, or on a modified cholic acid where a side chain with a free carboxyl group had been incorporated. The carboxyl groups on DHPTA must be protected to avoid carbamic anhydride formation resulting from reaction with the isocyanate. Reaction of the isocyanate with the tetraester-alcohol would lead to the carbamate linkage and removal of the protecting groups would give the final product. This is depicted in the scheme 3.14.

Scheme 3.14

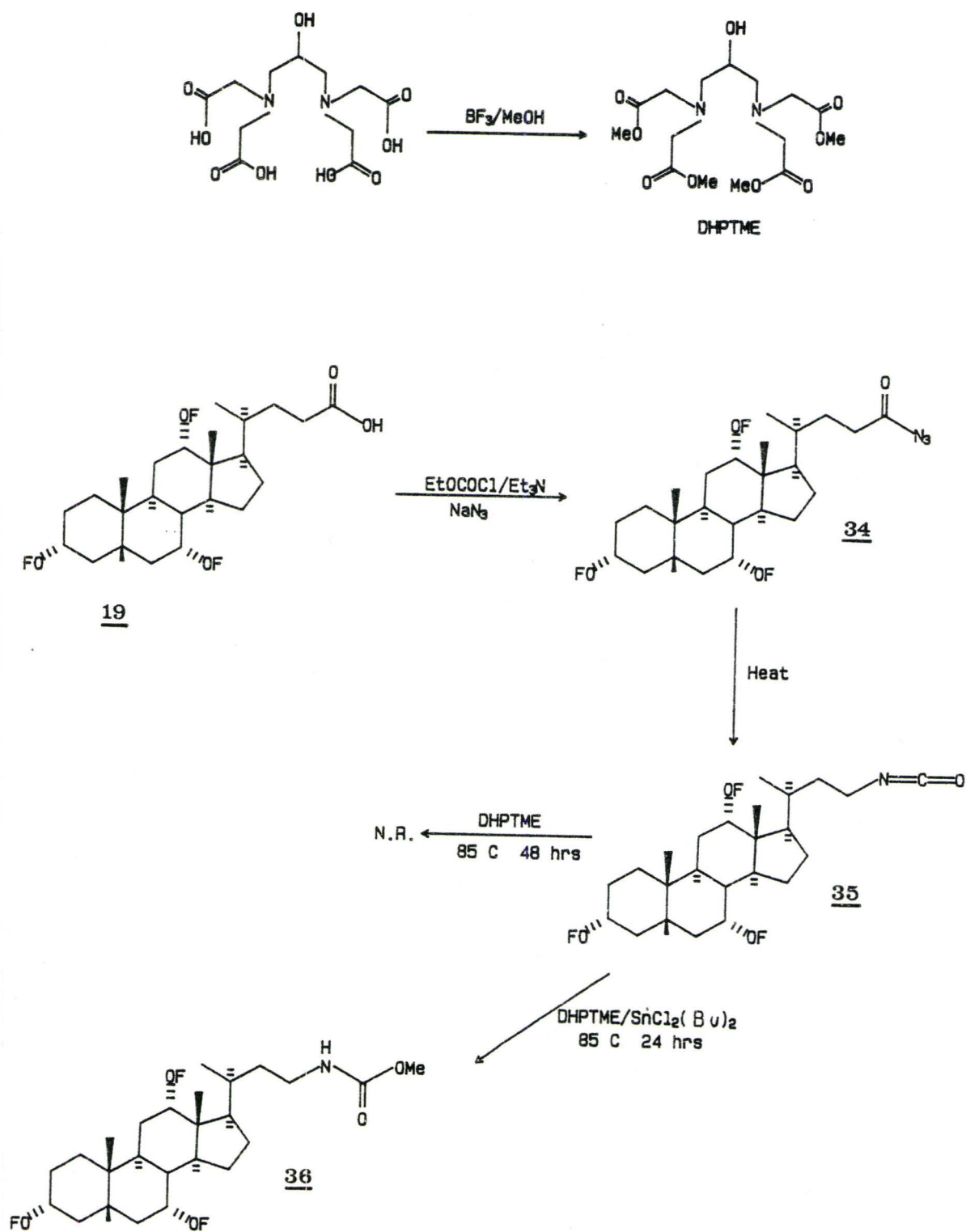


3.4.2 Synthesis

The isocyanate was prepared by treating cholic acid triformate with ethylchloroformate (a standard peptide coupling reagent) and sodium azide; the azide was heated to effect the Curtius rearrangement (March, 1985). The acid azide (34) and isocyanate (35) were not normally isolated but were characterized in preliminary studies. Proton NMR spectra in both cases remained substantially the same as the starting acid except for a slight shift in the methylene alpha to the carboxyl group (down field ≈ 0.5 ppm). IR spectra showed very intense bands at 2140 cm^{-1} for the acid azide and 2260 cm^{-1} for the isocyanate and both compounds were found to be ultraviolet (UV) sensitive on TLC plates under a 254 nm lamp.

The four carboxyl groups of DHPTA were protected by synthesizing the tetramethyl ester (18) using Lewis acid catalysis with boron trifluoride in methanol. The tetramethyl ester provided an interesting second order proton NMR spectrum. An ABX pattern was observed at 2.56 and 2.87 ppm as well as AB patterns at 3.56 and 3.63 ppm. Mass spectrometry gave a molecular ion at 378.

Coupling of the isocyanate and DHPTA tetramethyl ester was attempted by mixing the acid azide and hydroxy tetra-ester in an inert solvent and heating at 80°C . Heating was continued for 2 days with no appreciable amount of product formation. Formation of the isocyanate was indicated



by the evolution of nitrogen gas and confirmed by a strong band at 2260 cm^{-1} in the infrared spectrum of the product. It was concluded that the isocyanate was not reactive enough under these conditions and a new procedure be adopted.

It has been shown (Britain and Gemeinhardt, 1960) that several metal based compounds act as catalysts and greatly increase rates of reaction between isocyanates and hydroxyl groups. Tin compounds, in particular dibutyltin dichloride greatly increased reaction rates. The reaction was therefore carried out under conditions identical with those previously attempted, but with the addition of dibutyltin dichloride after the Curtius rearrangement was complete. TLC showed the formation of a single intense spot and at the same time showed the absence of the isocyanate. The major product was recovered by chromatography and was found to be the methyl carbamate (36). The production of this compound was attributed to the presence of methanol in the mixture as a result of the Lewis acid assisted cyclization of DHPTA tetramethyl ester to lactone 37.

Literature documentation (Burkus and Eckert, 1958) revealed that aromatic isocyanates are many times more reactive than aliphatic isocyanates. A trial experiment using phenyl isocyanate and 18 (with no catalyst present) was performed and reaction went to near completion in 20 minutes under identical conditions as those used in the previous attempts.

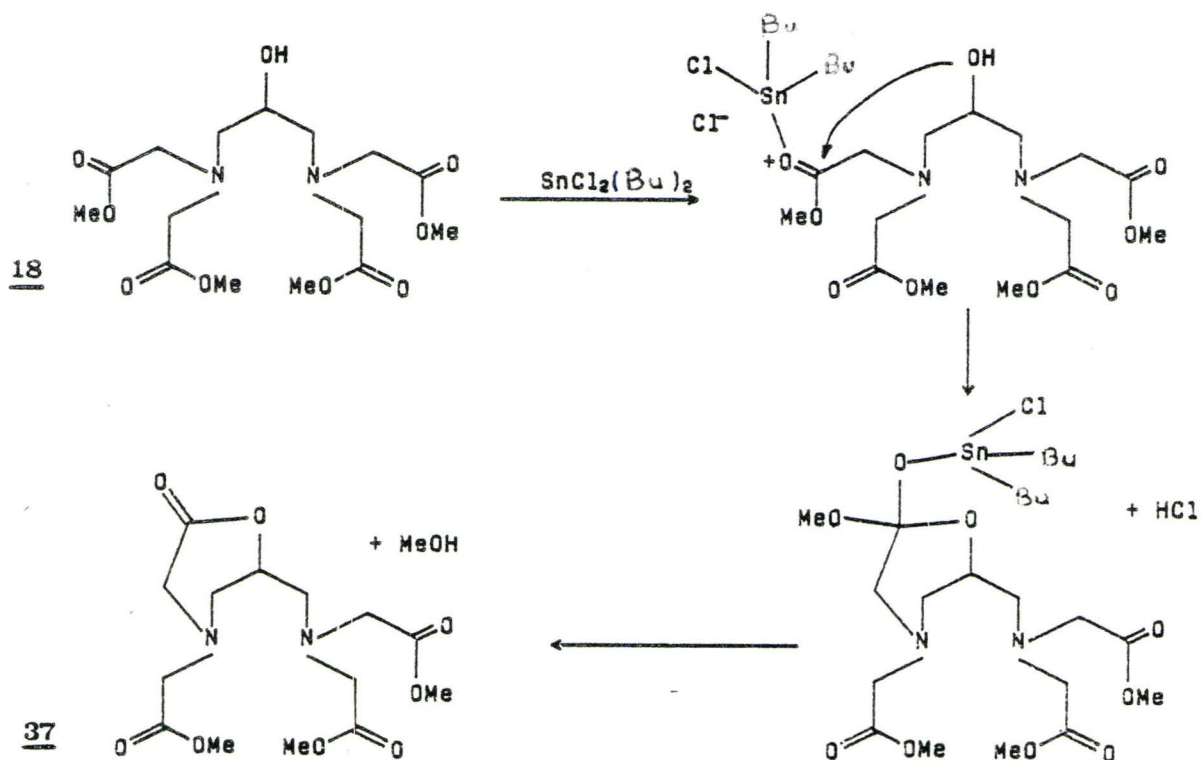


Fig 3.14 Mechanism of Lewis acid assisted cyclization of 18.

The greater reactivity of the aromatic isocyanate is attributed to the electron withdrawing effects of the aromatic ring and to the resonance stabilization of the intermediate.

Incorporation of a short aromatic carboxylic acid onto cholic acid was therefore undertaken. This was achieved by formation of the para-aminobenzoic acid amide of 19 with the use of carbonyl diimidazole as the peptide coupling

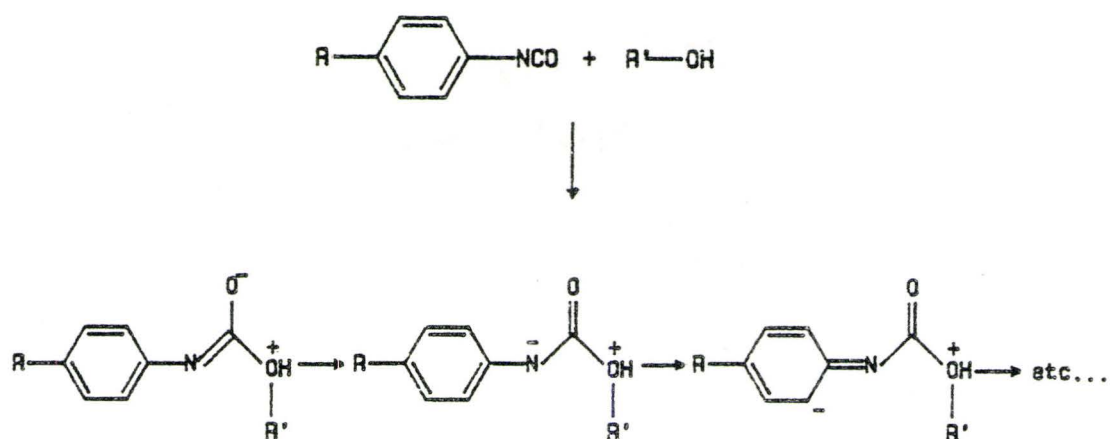
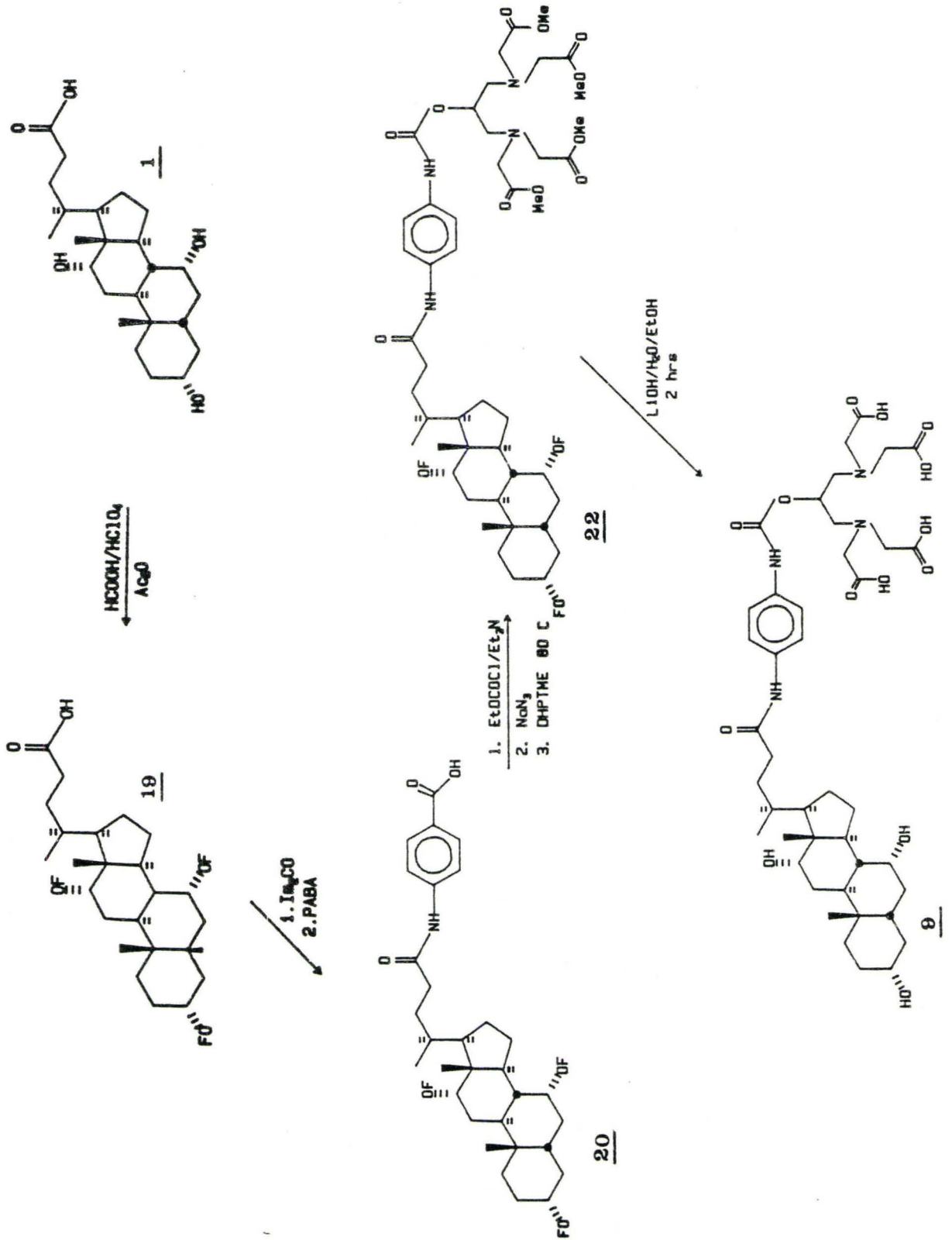


Fig 3.15 Resonance stabilization of the intermediate in the reaction of an aromatic isocyanate with an alcohol.

reagent. Mass spectrometry of compound 20 showed a strong $M+1$ peak at 612 and the typical AB pattern for para-substituted benzene rings was evident in the aromatic region of the proton NMR spectrum, as well as a broad amide NH singlet at 7.4 ppm. Formation of the acid azide (21) of 20 followed by the Curtius rearrangement then led to a reactive isocyanate. Reaction with 18 was complete in 30 minutes once the isocyanate had been generated. The proton NMR spectrum of the product (22) showed the incorporation of the chelating moiety and an additional NH signal at 4.9 ppm from the carbamate. The chelating moiety showed the same typical ABX and AB patterns seen previously in 18 but slight chemical shift changes altered line intensities and splittings noticeably.

Scheme 3.16



In the mass spectrum there was a strong molecular ion at m/z 987. Hydrolysis of the protecting groups of 22 using aqueous lithium hydroxide in ethanol (Hertzberg and Dervan, 1984) followed by acidification produced the final product. The mass spectrum of 9 showed a strong $M+1$ peak at m/z 847. The proton NMR spectrum confirmed that hydrolysis was complete by the absence of the signal at 3.67 ppm (methyl esters) and the absence of the three formate signals at approximately 8 ppm. The protons on carbons 3, 7, and 12 had shifted upfield from their position in compound 22.

3.4.3 Other Trial Experiments Towards Cholic Acid DHPTA

Before deciding to use a carbamate linkage to join cholic acid and DHPTA, two other approaches were explored. The simplest method for linking cholic acid to the chelating moiety is through an ester linkage. An ester linkage has the disadvantage that it undergoes hydrolysis easily but is normally easily formed and was therefore investigated. The hydroxyl groups on cholic acid were first protected by making the triformate (19) as described earlier. The carboxyl groups on DHPTA were protected by the formation of the tetramethyl ester (18). It was apparent even at this point that the hydroxyl group was quite hindered and therefore a highly activated carboxylic acid was required. Methane sulphonyl chloride was used as an activating agent to prepare the mixed sulphonic anhydride (Chandrasekaran

and Turner, J.V., 1982). However reaction of the mixed anhydride with the protected DHPTA did not occur as evidenced by TLC and proton NMR (only starting material present in both) and this approach was therefore abandoned.

Another approach was to form a secondary amine by converting the hydroxyl to a good leaving group, and displacing it with a primary amine. The tosylate was chosen as the leaving group. Attempts to prepare the tosylate were unsuccessful because pyridine used in the reaction caused cyclization of the tetraester to the lactone (37). The use of different carboxyl protecting groups would therefore be required to prepare the tosylate. Even then the tosylate may be inaccessible to nucleophilic attack because of the large steric hindrance of the carboxyl side chains. This approach was abandoned.

3.5 Two Dimensional NMR Analysis of Cholic Acid Triformate

Since the development of NMR there has been a problem with the assignment of proton NMR spectra for large molecules such as steroids. Aliphatic steroids like cholic acid and its derivatives are particularly difficult due to the large number of signals in the aliphatic region (approximately 1 to 2 ppm). Modern NMR techniques such as two dimensional (2-D) homonuclear and heteronuclear NMR can be used to assign chemical shifts to proton spectra if the carbon spectra have been previously assigned. Conversely, if

Fig 3.16 ^1H NMR spectrum for Cholic acid triformate

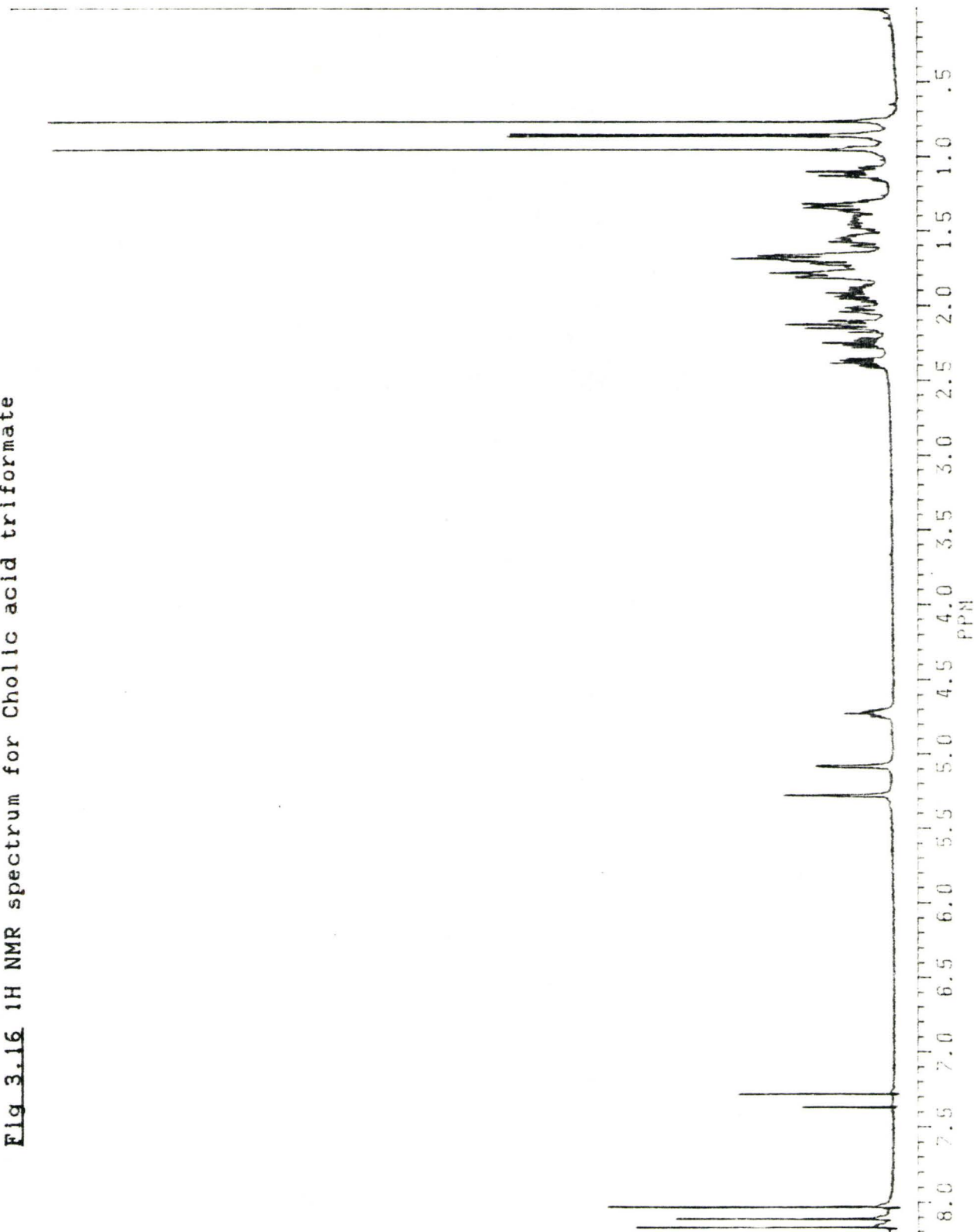
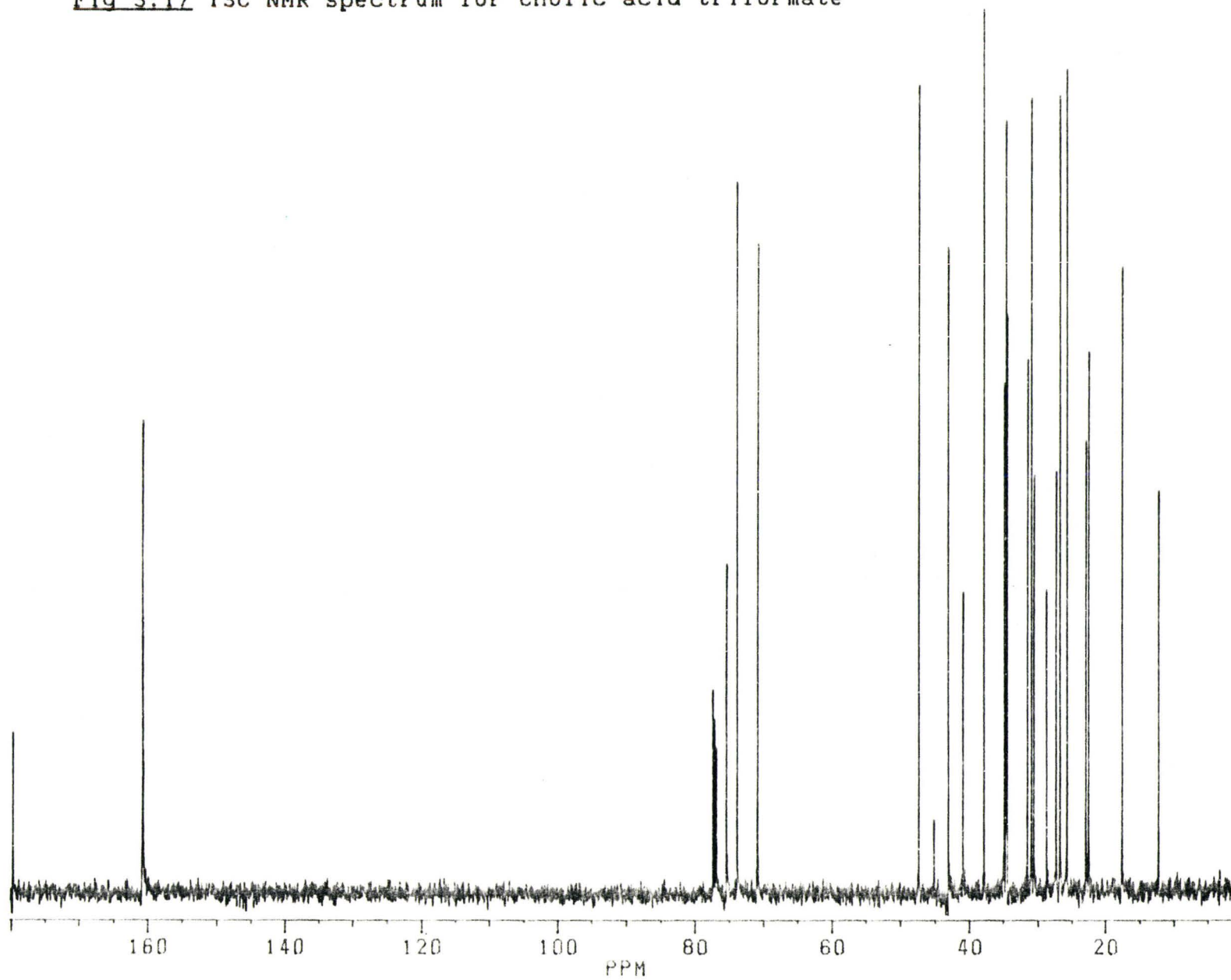


Fig 3.17 ^{13}C NMR spectrum for Cholic acid triformate



the proton spectra have been assigned, the carbon spectra can be assigned using these techniques (Derome,1987). Cholic acid triformate (1) has been examined in detail using these techniques. The carbon spectrum has been assigned by comparison to a documented spectrum of methyl cholate triacetate (Bremser et al.,1981) and with the use of spin sort C13 experiments which distinguish quaternary C's and CH₂'s from CH's and CH₃'s. The normal C13 spectrum can be seen in Fig 3.17. The proton spectrum (Fig 3.16) has a small number of assignable signals but for the most part is undecipherable due to the large number of overlapping multiplets.

3.5.1 Homonuclear 2-D NMR

The homonuclear 2-D spectrum (Fig 3.18) was run over only a small sweep width (0 to 5.8 ppm) to increase resolution. The spectrum does not include the formate singlets which were omitted as they show no coupling and would not provide any information. Some assignments have been made from the proton spectrum alone and these were used as starting points to obtain new information. The methyls for example have been assigned on the basis of the proton spectrum alone. The C18 and C19 methyls are singlets and show no cross peaks on the 2-D spectrum while the C21 methyl appears as a doublet and its coupling to H20 can be seen by a cross peak at 1.43 ppm corresponding to the chemical shift

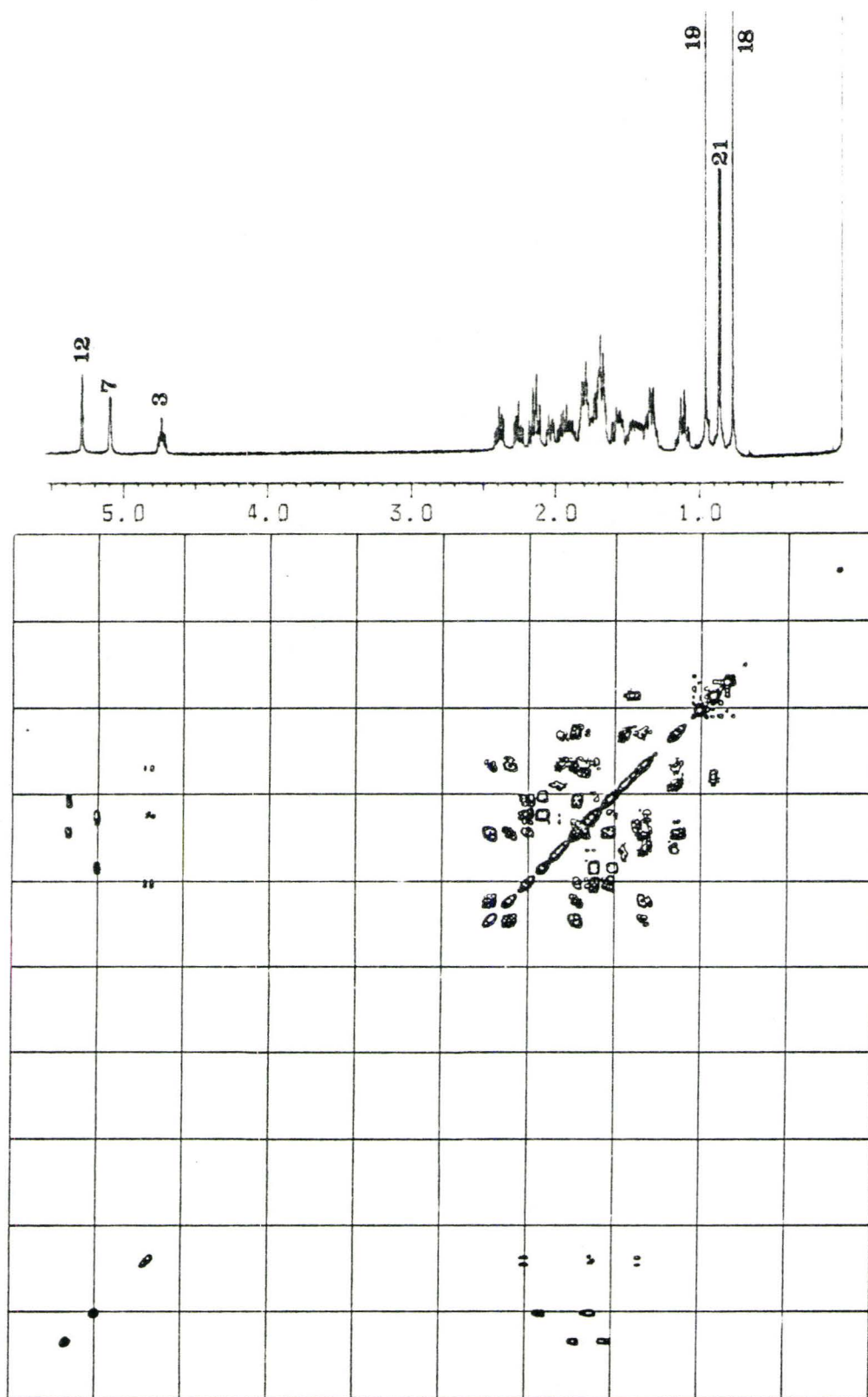


Fig 3.18 Homonuclear 2-D NMR spectrum for Cholic acid triformate

of H20. As another example, the proton on C12 is coupled to two protons on C11 and appears as a triplet in the proton spectrum at 5.27 ppm. The 2-D spectrum shows 2 cross peaks corresponding to chemical shifts at 1.60 and 1.78 ppm which can be tentatively assigned to the protons on C11; it is not possible using these techniques to determine which is H11 α and H11 β . However, for cases such as this, where the protons are situated on a six membered ring, it has been shown that the equatorial protons are generally further downfield by approximately 0.5 ppm (Bovey,1969) and assignments can be made based on this observation. Both C11 protons should show coupling to H9 α and the 2-D spectrum shows two cross peaks linking the H11 signals to H9 α at 2.13 ppm. This line of attack can be continued because H9 α is coupled to H8 β which is in turn coupled to other protons, and so on. Once this pathway has been exhausted another can be started at H3 and H7 until all the available information has been obtained and most of the chemical shifts have been tentatively assigned.

3.5.2 Heteronuclear 2-D NMR

The heteronuclear 2-D spectrum (Fig 3.19) provides much more information than the homonuclear spectrum and can be used to verify the assignments made previously. Carbon signals corresponding to quaternary carbons are not apparent in the spectrum since they show no proton coupling. Other assignments can be made directly. For example the three

methyl carbons, C18, C19 and C21, show one cross peak each corresponding to protons H18, H19 and H21, respectively. The next carbon peak downfield is C15 and it shows two cross peaks at 1.12 and 1.45 ppm on the proton axis corresponding to H15_α and H15_β. Again, this technique alone can not determine which proton is which. Carbon 11 is next downfield and is correlated to two protons with chemical shifts at 1.60 and 1.78 ppm (H11_α and H11_β) which verifies assignments obtained from the homonuclear 2-D spectrum. After examining the entire spectrum and cross referencing the chemical shifts obtained to those from the homonuclear spectrum, a set of proton chemical shifts can be generated as shown in Fig 3.20.

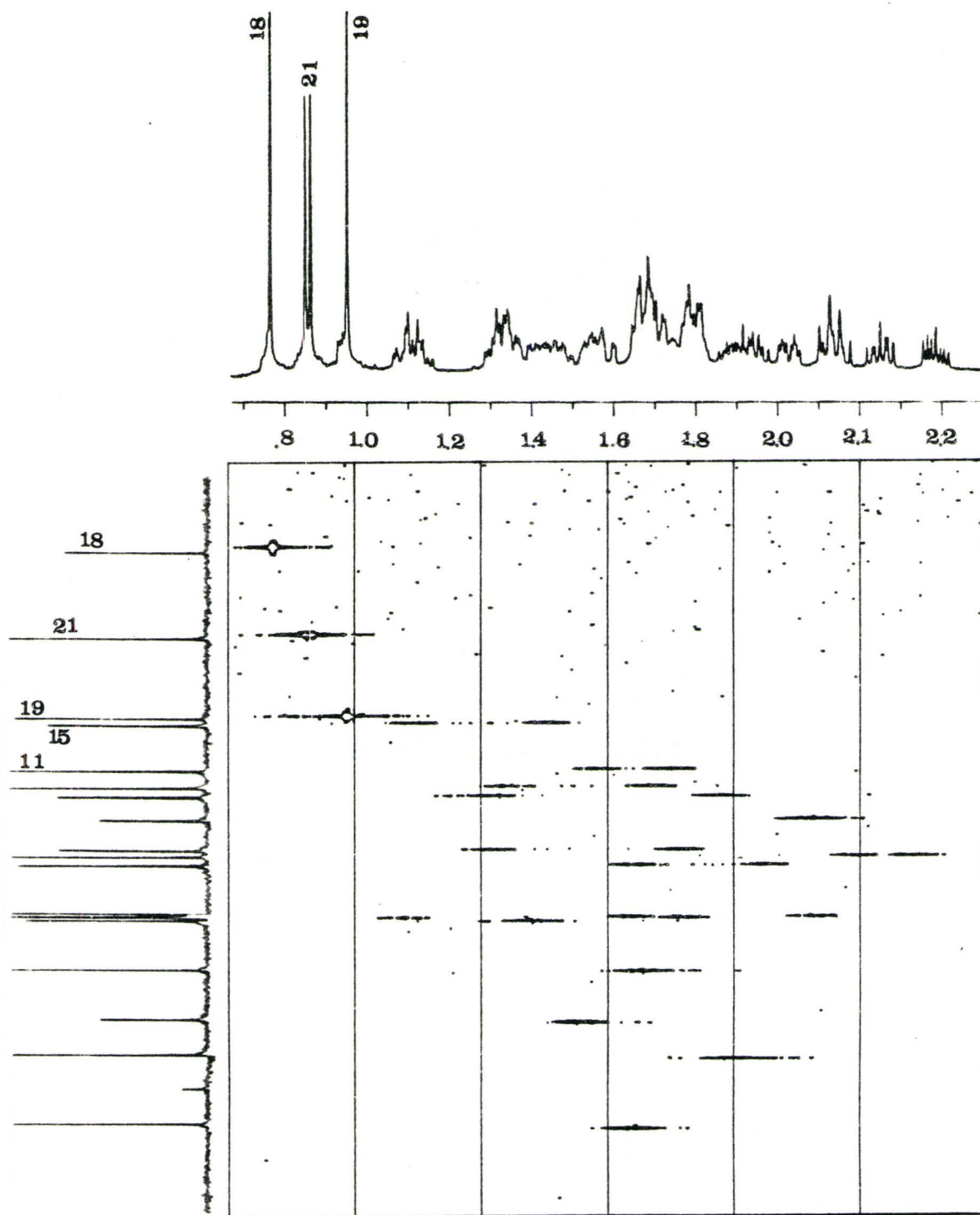


Fig 3.19 Heteronuclear 2-D NMR spectrum for Cholic acid triformate

Proton Chemical Shifts Acquired from Two Dimensional
NMR Analysis of Cholic Acid Triformate

<u>Proton</u>	<u>Chemical Shift</u>
1 α	1.09
1 β	1.81
2 α	1.73
2 β	1.37
3 β	4.72
4 α	2.14
4 β	1.67
5 β	1.55
6 α	1.69
6 β	2.02
7 β	5.08
8 β	1.71
9 α	2.13
11 α	1.60
11 β	1.78
12 β	5.27
14 α	1.96
15 α	1.12 a
15 β	1.45 a
16 α	1.33 b
16 β	1.90 b
17 α	1.68
18(CH ₃)	0.76
19(CH ₃)	0.95
20	1.43
21(CH ₃)	0.85
22	1.32 c
22'	1.79 c
23	2.20 d
23'	2.38 d
24(COOH)	10.16
25	8.02
26	8.11
27	8.16

Note: a, b, c, d, chemical shifts may be reversed for any given letter

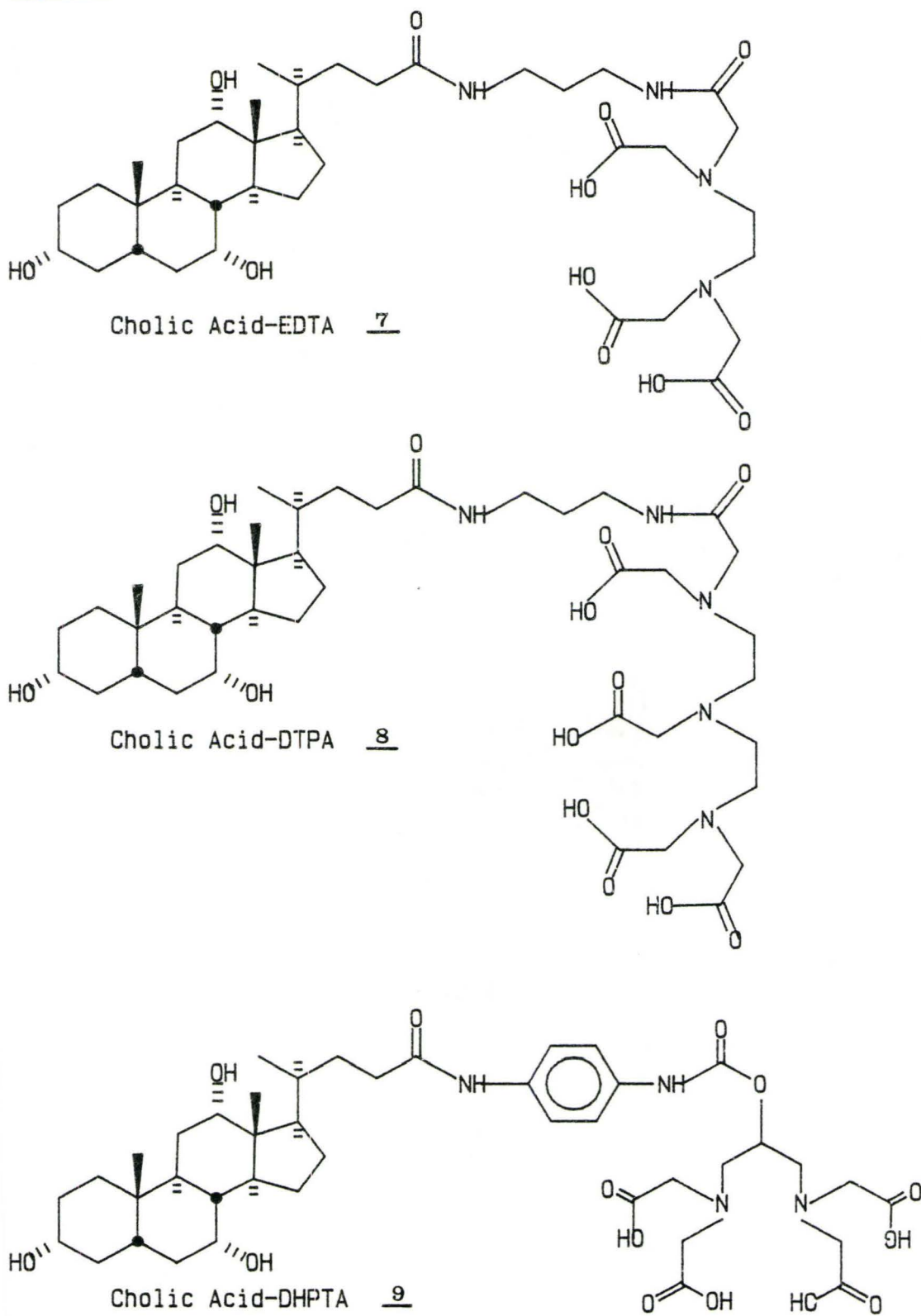
3.6 Future Experiments

To be used as biological probes the three chelating reagents must be chelated to metal ions. The chelate complexes must then be examined to determine their usefulness before being used in vivo. First and foremost the stability constants of the metal chelate complexes must be determined; a low stability constant implies that the complex is unsuitable for use in biological systems. The solubility product should be determined and if possible should be determined in a simulated biological medium to ensure the complex remains in solution when used in a biological system. Next the spectroscopic properties of the metal complex may need to be considered. For example, if a paramagnetic metal ion is complexed, its NMR relaxation effects can be monitored in vitro. If the metal has other spectroscopic properties which may be altered by chelation (eg: luminescent energy transfer) these also should be examined in vitro. Finally, the metal complexes, if they have the desired physical properties should be tested in vivo.

CONCLUSIONS

This thesis has concerned itself with the synthesis of three chelating reagents derived from cholic acid: cholic acid-EDTA, cholic acid-DTPA and cholic acid-DHPTA as shown in Fig 4.1. The syntheses were carried out using the chelating moieties as starting materials. Cholic acid-EDTA and cholic acid-DTPA were prepared by the conversion of cholic acid to a primary amine by the addition of a diamine spacer chain, followed by amide formation at one carboxyl site of the activated EDTA and DTPA species, respectively. Cholic acid-DHPTA was prepared by first attaching p-aminobenzoic acid to the free cholic acid side chain. Conversion of the adduct to the aryl isocyanate and reaction with the hydroxyl group on DHPTA produced a carbamate and led to the final product. All three compounds were fully characterized and are ready for testing as chelating reagents with different metal ions, and eventually for testing in vivo.

Fig 4.1 Summary of the three final biological chelates



EXPERIMENTAL

General Introduction

Proton nuclear magnetic resonance spectra (^1H NMR) were recorded on Varian EM390, Bruker WP80, Bruker WM250, and Bruker AM500 nuclear magnetic resonance spectrometers at 90, 80, 250 and 500 MHz, respectively. When describing the spectra the terms s=singlet, d=doublet, t=triplet, q=quartet and m=multiplet refer to the multiplicities of the signals observed. Natural abundance carbon thirteen NMR (^{13}C NMR) were recorded on Bruker WM 250 and Bruker AM 500 nuclear magnetic resonance spectrometers at 62.873 and 125.776 MHz, respectively and were broad band decoupled. Two dimensional heteronuclear and homonuclear NMR spectra were recorded on the Bruker AM 500. All NMR spectra were acquired using deuterated solvents. Deuterated methanol, dimethyl sulfoxide, and chloroform (MeOH-d_4 , DMSO-d_6 , and CDCl_3 , respectively) were used and are noted for each spectrum. Tetramethylsilane (TMS) was used as the internal reference.

Infrared spectra, when required were recorded on a Perkin Elmer 283 IR Spectrophotometer using liquid cells with dry chloroform as the solvent. For insoluble compounds, potassium bromide pellets were prepared. All spectra were calibrated using a polystyrene film. The terms s=strong, m=medium, w=weak were used in describing particular bands of

interest.

Mass spectra were recorded on a VG ZAB-E mass spectrometer by R.W. Smith and co-workers. The terms EI, CI, and FAB (electron impact, chemical ionization, and fast atom bombardment, respectively) refer to the technique used in acquiring spectra and are noted in each case.

Where chromatography was required, flash chromatography was performed by the method of W.C. Still (1978) using Silica gel 60 (230-400 mesh ASTM). TLC were performed using silica gel 60 F254 (E. Merck Co.) plates. Spots were visualized using a UV lamp at 254 nm. Where this was not possible spots were visualized using Ninhydrin or Molybdate spray reagents (Dawson et al, 1969).

When dry solvents or liquid reagents were required they were distilled by literature procedures. Pyridine was distilled from calcium hydride. Triethylamine and N-methyl morpholine were distilled from 1% phenyl isocyanate. Dioxane, tetrahydrofuran, and benzene were distilled from sodium and benzophenone. Methanol was distilled from magnesium. Ethyl acetate and chloroform were passed through an alumina column directly into the reaction vessel.

Melting points were recorded using a Gallenkamp melting point apparatus and are uncorrected. All yields quoted are for pure isolated products.

Synthesis of EDTA Dianhydride (13)

This synthesis was performed according to methods previously described (J.R. Geigy, 1968). EDTA (1.8 g, 0.006 mole) was suspended in 3 g of dry pyridine under an inert atmosphere. Acetic anhydride (2.6 g) was then added and the solution was left stirring vigorously at 65°C for 24 hrs. At this time the solution was vacuum filtered under a steady stream of dry nitrogen and washed with acetic anhydride (20 ml) and diethyl ether (20 ml). The resulting white solid (1.45g, 94 %) was left under high vacuum for 24 hrs and then stored in a desiccator (mp. 194-195°C).

MS(EI): m/z (RI%); 256(7)[M⁺], 229(6), 184(4), 141(21),

128(100)[symmetrical cleavage], 56(60)

IR: ν_{\max} ; 1770(s)[anhydrides], 2900(w)

¹H NMR: δ 2.670(s, 4H, N-CH₂-CH₂-N), 3.710(s, 8H, N-CH₂-C=O-) (DMSO-d₆, 500 MHz)

¹³C NMR: δ 50.72(N-CH₂-CH₂-N), 51.79(N-CH₂-C=O-), 165.26(N-CH₂-C=O-) (DMSO-d₆, 500 MHz)

Synthesis of EDTA Monoanhydride (14)

This procedure was cited in a previous publication (T. Takeshita et al., 1982). Compound 13 (1.3 g, 0.005 mole) was suspended in 8 ml of dry DMF and heated until dissolution was apparent. The temperature was adjusted to 70-72°C and water (0.090 ml, 0.005 mole) was added dropwise.

The solution was stirred at this temperature for 3 hrs over which time the monoanhydride (14) precipitated. The reaction mixture was cooled to room temperature. The precipitate was vacuum filtered under a steady stream of dry nitrogen and washed with dry ether (25 ml). The white solid was collected and dried under high vacuum to yield 1.2 g (81%) of 14 (decomp. 188-190°C).

MS(EI): m/z (RI%); 274(1)[M⁺], 256(3)[M⁺-H₂O],
216(4)[M⁺-CH₂COO], 187(12), 171(98)[216-COOH],
146(21)[NCH₂-CH₂N cleavage, larger fragment],
128(100)[other half of 146], 143(46), 56(83)

IR: ν_{\max} ; 1640(m)[carboxyls], 1770(s)[anhydride], 2950(w)

¹H NMR: δ 2.595(t, J=6 Hz, 2H, N-CH₂-CH₂-N on carboxyl side), 2.802(t, J=6 Hz, 2H, N-CH₂-CH₂-N on anhydride side), 3.431(s, 4H, CH₂ next to COOH), 3.725(s, 4H, CH₂ next to anhydride) (DMSO-d₆, 500 MHz)

¹³C NMR: δ 50.56(C_A), 52.05(C_B), 52.50(C_C), 54.64(C_D),
165.67(anhydride carbonyls), 172.35(carboxyl carbonyls) (DMSO-d₆, 500 MHz)

Synthesis of DTPA Dianhydride (17)

This procedure was cited previously (J.R. Geigy, 1968). DTPA (3.1 g, 0.0078 mole) was suspended in a mixture of acetic anhydride (4.9 g) and dry pyridine (4.1 g) under an inert atmosphere and warmed to 65°C. The suspension was

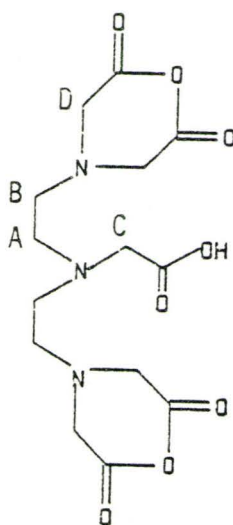
stirred vigorously for 48 hrs. The reaction mixture was cooled to room temperature, filtered by vacuum under a dry stream of nitrogen and washed with acetic anhydride (40 ml) and dry benzene (30 ml). The cream coloured solid (2.5 g, 90 %) was left under high vacuum for 24 hrs and stored in a desiccator (mp. 169-171°C).

MS(EI,CI,FAB): Not Obtainable

IR: ν_{\max} ; 1640(w)[carboxyl], 1760(s)[anhydrides]

$^1\text{H NMR}$: δ 2.593(t, $J=7.5$ Hz, 4H, H_A), 2.744(t, $J=7.5$ Hz, 4H, H_B), 3.304(s, 2H, H_C), 3.712(s, 8H, H_D)
(DMSO- d_6 , 500 MHz)

$^{13}\text{C NMR}$: δ 50.67(C_A), 51.73(C_B), 52.53(C_C), 54.47(C_D), 165.67(anhydride carbonyls), 171.89(carboxyl carbonyl) (DMSO- d_6 , 500 MHz)



Synthesis of Methyl Cholate (15)

Cholic acid (3 g, 0.0073 mole) was introduced to a dry 100 ml round bottom flask equipped with a condenser and under positive nitrogen pressure. Dry methanol (45 ml) was then added followed by 12 ml (excess) boron trifluoride-methanol complex (50 wt.%). The solution was refluxed for 2 hrs, cooled to room temperature and then poured with vigorous stirring into 75 ml of saturated sodium bicarbonate at 0°C and was extracted (3 times 25 ml) with chloroform. The chloroform layers were combined and evaporated to dryness on a rotary evaporator to produce a white solid. Recrystallization from methanol yields 2.8 g (90 %) colourless needles (mp. 148-149°C).

MS(FAB): m/z (RI%); 423(4)[M+1], 405(3.5)[M+1-H₂O],
387(13)[M+1-2H₂O's], 369(15)[M+1-3H₂O's], 185(50),
93(100), 75(35)

TLC: (90:10 / ethyl acetate:hexane) R_f=0.20

¹H NMR: δ 0.688(s, 3H, C-18 CH₃), 0.894(s, 3H, C-19 CH₃),
0.984(d, J=6.4 Hz, 3H, C-21 CH₃), 3.453(m, 1H, C3-H),
3.665(s, 3H, -COOCH₃), 3.851(unresolved q, 1H, C7-H),
3.974(unresolved t, 1H, C12-H),
0.945 to 2.405(overlapping m, 27 remaining protons)
(CDCl₃, 500 MHz)

¹³C NMR: ppm(C#); 12.51(18), 17.31(21), 22.50(19),
23.18(15), 26.59(9), 27.42(16), 28.30(11), 30.52(2),

30.89(23), 31.05(22), 34.61 and 34.69(6+10),
 35.17(20), 35.23(1), 39.57(8), 39.69(4), 41.46 and
 41.86(5+14), 46.48(13), 47.11(17), 51.59(25),
 68.40(7), 71.94(3), 72.97(12), 174.72(24) (CDCl₃,
 500 MHz)

Synthesis of Cholic acid 1,3-Diaminopropane Amide (16)

Compound 15 (700 mg, 0.0017 mole) was dissolved in 5 ml of 1,3-diaminopropane in a 20 ml round bottom flask fitted with reflux condenser. The solution was refluxed for 4 hrs at which time the solvent was removed under high vacuum on a rotary evaporator to yield a cream coloured foam. The foam was dissolved in a minimum amount of methanol and flash chromatographed (1 % conc. ammonia in methanol) on silica to yield 631 mg (82%) of white solid (mp 156-158°C).

MS(FAB): m/z(RI%); 465(30)[M+1], 447(100)[M+1-H₂O],
 429(25)[M+1-2H₂O's], 185(54), 93(94)

TLC: (1% (v/v) NH₃ in methanol) R_f=0.25

¹H NMR: δ 0.703(s, 3H, C18-CH₃), 0.910(s, 3H, C19-CH₃),
 1.024(d, J=6.5 Hz, 3H, C21-CH₃), 2.653(t, J=6.8 Hz,
 2H, -CH₂NH₂), 3.224(t, J=6.8 Hz, 2H, -CH₂NHC=O-),
 3.367(m, 1H, C3-H), 3.789(unresolved q, 1H, C7-H),
 3.942(unresolved t, 1H, C12-H), 0.951 to
 2.400(overlapping m, 32 remaining protons)
 (MeOH-d₄, 500 MHz)

^{13}C NMR: ppm(C#): 13.04(18), 17.77(21), 23.22(19),
 24.22(15), 27.84(9), 28.70(16), 29.59(11), 31.19,
 33.27, 33.35, 34.13, 35.90, 36.52, 36.92(20), 37.55,
 39.65, 40.46, 41.01(8), 42.97+43.17(5+14),
 47.46(13), 47.98(17), 68.98(7), 72.84(3), 73.98(12),
 173.93(24) (MeOH- d_4 , 500 MHz)

Synthesis of Cholic Acid-EDTA (7)

EDTA monoanhydride (177 mg, 0.645 mmole) was introduced to a dry reaction vessel under a nitrogen atmosphere. Dry DMF (8 ml) was introduced and the solution cooled to 0°C. To this, 2 ml dry DMF containing 16 (300 mg, 0.645 mmole) was added and the resulting solution stirred at 0°C for 15 min. The solvent was removed under reduced pressure on a rotary evaporator and the residue was purified by flash chromatography on silica (50:50 / ethanol:methanol) to yield 397 mg (84%) of white solid (decomp. 213-215°C).

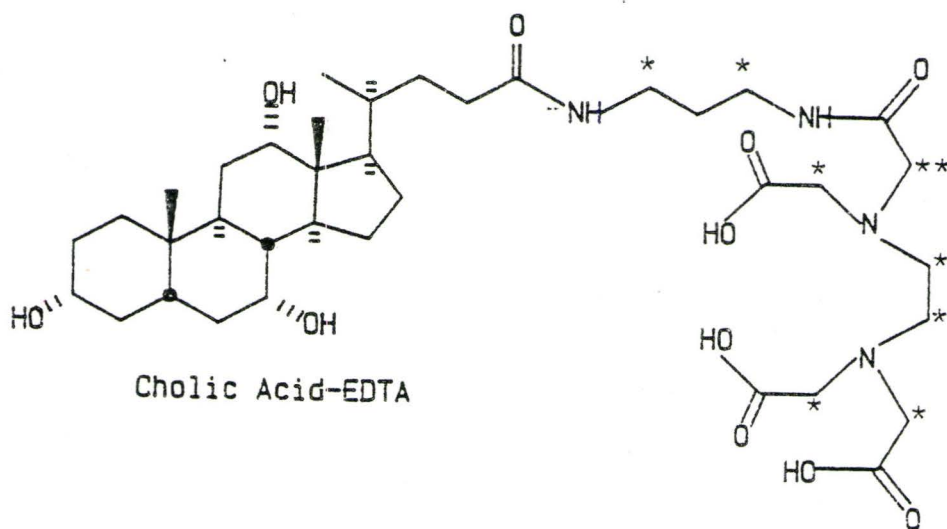
MS(FAB+): m/z (RI%); 739(100)[M+1], 681(66)[M+1-CH₂COO],
 623(19)[M+1-2 CH₂COO's], 515(21), 461(85), 423(63)

TLC: (100% methanol) R_f=0.53

^1H NMR: δ 0.691(s, 3H, C18-CH₃), 0.923(s, 3H, C19-CH₃),
 1.030(d, J=6.5 Hz, 3H, C21-CH₃), 2.605(m, 2H, H**),
 3.369(m, 1H, C3-H), 3.786(unresolved q, 1H, C7-H),
 3.937(t, 1H, C12-H), 2.937 to 3.316(m, 10H, H*'s),
 0.857 to 2.361(overlapping m, 38 remaining protons)

(MeOH-d₄, 500 MHz)

¹³C NMR: ppm(C#); 13.07(18), 17.84(21), 23.22(19),
 24.28(15), 27.94(9), 28.79(16), 29.64(11), 30.17,
 31.24(2), 33.37, 34.17, 35.94, 36.55, 37.01(20),
 37.98, 38.40, 40.54, 41.08(8), 43.06 and
 43.25(5+14), 47.53(13), 48.05(17), 56.78 and
 56.98(30+31) 60.76(29), 62.21 and 62.81 and
 63.26(32+34+36), 69.06(7), 72.93(3), 74.03(12),
 174.99 and 176.94(24+28), 179.74 and 179.83 and
 180.20(33+35+37) (MeOH-d₄, 500 MHz)



Solid Phase Synthesis of Cholic acid-EDTA (7)

Synthesis of Hydroxy Resin (HO-R)

The resin was synthesized by methods previously reported (Stewart and Young, 1969). In a round bottom flask with condenser and nitrogen atmosphere 2 g of 1% crosslinked (200-400 mesh, 1.2 mequiv. Cl/g) polystyrene divinylbenzene copolymer (Aldrich) was suspended in 12 ml of dry 2-methoxyethanol. Potassium acetate (0.259 g, 1.1 equiv.) was added and the mixture heated at 130°C for 24 hrs. The reaction mixture was cooled, and the acetylated resin (AcO-R) filtered and washed with water (30 ml) and methanol (30 ml) and allowed to dry under high vacuum.

IR: ν_{\max} ; 1735(s)[ester]

Hydrolysis of AcO-R was performed by suspending 2 g of acetylated resin in 14 ml of ortho-dichlorobenzene (ODCB) and adding 5 g potassium hydroxide in 5 ml water, and 0.25 ml of 40% tetrabutylammonium hydroxide. The solution was heated at 75°C for 20 hrs. The resulting hydroxy resin (HO-R) was filtered and washed with benzene, methanol, water, methanol and ether (50 ml of each) in that order and dried under high vacuum.

IR: ν_{\max} ; 3440(m)[OH's], 3560(m)[OH's]

Attachment of 13 onto Hydroxy Resin to form 13-R

Compound 13 (278 mg, 0.0011 mole) was dissolved in 6 ml of dry DMF in a dry Schlenk tube apparatus (Fyles and Leznoff, 1976) under nitrogen atmosphere. To this was added 0.25 g of HO-R and the resulting suspension stirred at 75°C for 12 hrs. The hot solution was filtered by suction while maintaining a nitrogen atmosphere. The resulting resin (13-R) was washed repeatedly with dry DMF and left in the apparatus for further reaction (a small portion was dried for IR analysis).

IR: ν_{\max} ; 1820(m)+1760(m)[anhydride C=O's], 1730(s)[ester C=O]

Attachment of Cholic Acid Diamino Propane Amide to 13-R to form 16-R

The resin 13-R was resuspended in 3 ml dry DMF at 25°C and 16 (135 mg, 0.00029 mole) was added. The mixture was stirred for 15 minutes and the product (16-R) filtered. It was washed with methanol, water, 0.25 M HCl (until no further amine was detected in the washings), water and methanol (a small portion was dried for IR analysis).

IR: ν_{\max} ; 3100-3500(w)[OH's], 1730(s)[ester C=O],
1640(w)[amide C=O]

Hydrolysis to give Cholic Acid-EDTA (7)

Resin 16-R (200 mg) was suspended in 10 ml methanol and 150 mg K_2CO_3 was added. The suspension was refluxed for 12 hrs. Water (2 ml) was added and the pH adjusted to 2 by addition of 1N HCl. The mixture was filtered and the filtrate evaporated. The resulting residue was flash chromatographed on silica (50:50 / ethanol:methanol) to produce 7 (41 mg, 21% calculated from 16).

IR of the resin was identical to starting hydroxy resin.

Synthesis of Cholic Acid-DTPA (8)

Compound 17 (321 mg, 0.898 mmole) was dissolved in 30 ml of dry DMF under an inert atmosphere and cooled to $-45^\circ C$ using a dry ice / acetonitrile bath. Compound 16 (140 mg, 0.299 mmole) dissolved in 40 ml DMF was added dropwise and the solution was stirred for an additional 30 min at $-45^\circ C$. Water (10 ml) was added and the solution was heated to $75^\circ C$ for 30 min. The amount of solvent was reduced to 3 ml and the resulting precipitate (DTPA) was removed by filtration. The remaining solvent was evaporated under vacuum and the residue was flash chromatographed (3:2:1 / ethanol: chloroform: water) to produce 105 mg of a white solid (42 %, decomp. at $200-210^\circ C$).

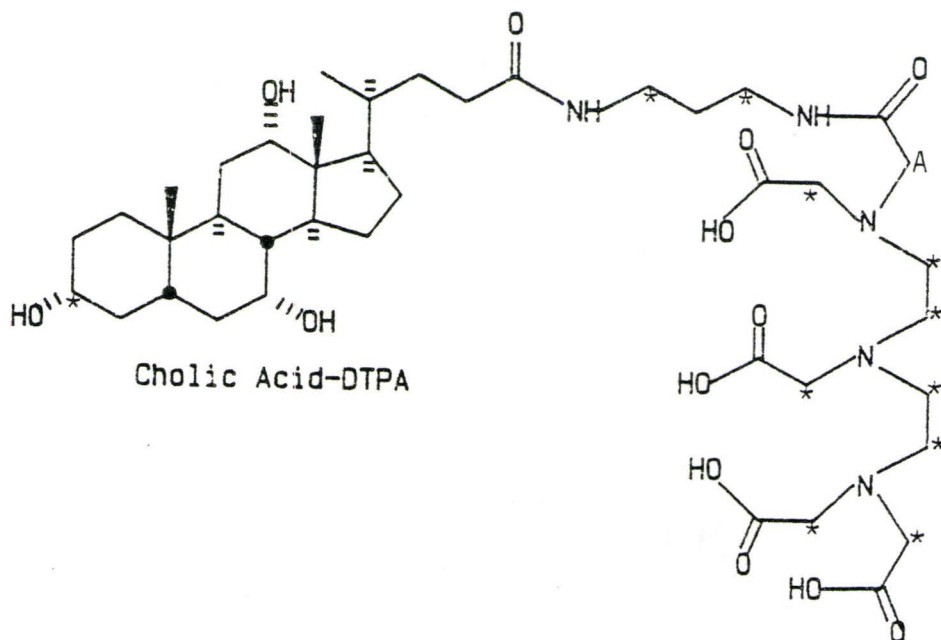
MS(FAB+): m/z (RI%); 840(100)[M+1], 782(80)[M+1- CH_2COO],

724(26)[M+1-2CH₂COO's], 681(17), 580(25), 507(47)

TLC: (3:2:1 ethanol:chloroform:water) R_f=0.3

¹H NMR: δ 0.682(s, 3H, C18-CH₃), 0.894(s, 3H, C19-CH₃),
1.015(d, J=6.5 Hz, 3H, C21-CH₃), 2.701(m, 2H, H_A),
3.060 to 3.394(overlapping m, 17H, H's on C*),
3.772(unresolved q, 1H, H on C7), 3.924(unresolved
t, 1H, H on C12), 0.924 to 2.379(overlapping m,
39 remaining protons) (MeOH-d₄, 500 MHz)

¹³C NMR: ppm(C#); 13.02(18), 17.79(21), 23.18(19),
24.22(15), 27.87(9), 28.69(16), 29.55(11), 30.02,
31.17, 33.30, 34.18, 35.88, 36.49, 36.91(20), 37.68,
40.45, 40.99, 42.99 and 43.17(5+14), 47.48(13),
47.99(17), 48.50, 49.51, 49.62, 51.04, 51.51, 53.40,
53.97, 54.17, 54.46, 56.22, 56.50, 56.61, 57.47,
58.76, 69.04(7), 72.86(3), 74.01(12), 170.54,
171.35, 172.80, 173.88, 174.34, 174.58(28),
176.97(24) (MeOH-d₄, 500 MHz)



DHPTA Tetramethyl Ester (18)

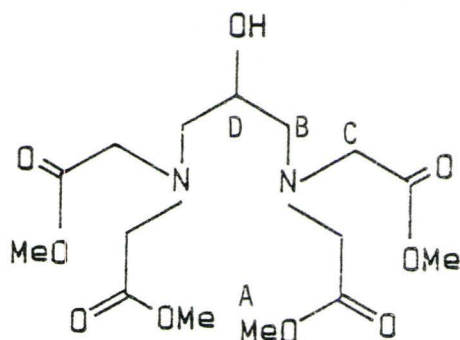
Under dry conditions and nitrogen atmosphere DHPTA (1 g, 0.0031 mole) was suspended in 15 ml dry methanol in a round bottom flask fitted with reflux condenser. Boron trifluoride methanol complex (2.7 ml, 50 wt. % solution) was added dropwise via syringe. At this time the reaction mixture became clear and the solution was refluxed for 90 minutes, cooled to 25°C and poured into 100 ml of saturated sodium bicarbonate at 0°C. The aqueous solution was extracted (3 times 60 ml) with chloroform. The organic layers were combined, dried with sodium sulfate and concentrated on a rotary evaporator to yield a clear colourless oil (1.01 g, 85.5%). This compound was found to decompose to compound 37 at -10°C in a benzene matrix over approximately 2 weeks.

MS(EI): m/z (RI%): 378(7)[M⁺], 346(10)[M⁺-CH₃OH],
 319(39)[M⁺-COOCH₃], 287(67)[319-CH₃OH], 200(37),
 174(96), 144(44), 116(100), 82(78)

TLC: (4:1/ethyl acetate:hexane) R_f=0.45

¹H NMR: δ 2.633(dd, J_{AB}=13.6 Hz, J_{AX}=8.5 Hz, 2H(part of ABX), H on C1), 2.879(dd, J_{AB}=13.6 Hz, J_{AX}=3.1 Hz, 2H(part of ABX), H on C1), 3.569(d, J_{AB}=17.7 Hz, 4H(part of AB), -CH_HCOOMe), 3.637(d, J_{AB}=17.7 Hz, 4H(part of AB), -CH_HCOOMe), 3.702(s, 12H, methyl esters), 3.733(m, 1H, H on C2) (CDCl₃, 500 MHz)

^{13}C NMR: δ 51.64(C_A), 55.90(C_B), 58.72(C_C), 67.95(C_D),
172.09(ester $\text{C}=\text{O}$) (CDCl_3 , 500 MHz)



Synthesis of 3,7,12-Triformyloxycolic Acid (19) (Cholic Acid Triformate)

This procedure is a modification of one previously reported (H.Takikawa et al, 1982). Cholic acid (5 g, 0.012 mole) was dissolved in 30 ml of 90% formic acid. To this was added 6 drops 70% perchloric acid and the solution was heated to 55-60°C for 1.5 hr. It was then cooled to 40°C and acetic anhydride was added dropwise until a large quantity of bubbles appeared (approx. 25 ml acetic anhydride). During this time the temperature increased and was maintained at 55-60°C. The solution was cooled to 25°C and poured into 200 ml of ice water. The precipitate was filtered and recrystallised from 90% ethanol to yield 5.1 g (84%) colourless crystals (mp. 196-197°C).

MS(FAB-): m/z (RI%) 491.3(24)[$\text{M}-1$], 463.3(100)[$\text{M}-1-\text{CO}$],
435(17)[$\text{M}-1-2 \text{ CO}'\text{s}$], 417(11)[435- H_2O], 275(13),

183(58), 137(15), 91(100)

TLC: (4:1/ethyl acetate:hexane) $R_f=0.74$

^1H NMR: δ 0.762(s, 3H, C18-CH₃), 0.853(d, J=6.3 Hz, 3H, C21-CH₃), 0.950(s, 3H, C19-CH₃), 4.719(m, 1H, C3-H), 5.076(q, J=3 Hz, 1H, C7-H), 5.270(t, J=3 Hz, 1H, C12-H), 8.023(s, 1H, C3-formate H), 8.106(s, 1H, C7-formate H), 8.162(s, 1H, C12-formate H), 10.160(broad s, 1H, -COOH), 1.058 to 2.171(overlapping m, 24 remaining protons) (CDCl₃, 500 MHz)

For further details see 2-D analysis.

^{13}C NMR: δ (C#) 12.14(18), 17.43(21), 22.32(19), 22.76(15), 25.54(11), 26.57(2), 27.13(16), 28.56(9), 30.41(23), 30.79(22), 31.32(6), 34.26(10), 34.44(1), 34.50(4), 34.70(20), 37.72(8), 40.78(5), 42.96(14), 45.02(13), 47.21(17), 70.67(7), 73.73(3), 75.26(12), 160.58(25,26,27), 179.56(24) (CDCl₃, 500 MHz)

Synthesis of Cholic Acid Triformate Para-Aminobenzoic Acid Amide (20)

To a solution of 19 (3 g, 0.0061 mole) in 6 ml of dry THF under an inert atmosphere at 0°C was added carbonyl diimidazole (1.1 g, 1.1 equiv.). The solution was stirred for 1 hr until the bubbling subsided and para-aminobenzoic acid (1.086 g, 1.3 equiv.) was added. Stirring was continued for 4 hrs and then the solvent was removed on a rotary

evaporator. The resulting solid was recrystallised from 50:50 ethanol:methanol to yield 2.9 g (78%) of colourless crystals (mp. 245-246°C).

MS(FAB+): m/z (RI%); 612(100)[M+1], 594(3)[M+1-H₂O],
584(5)[M+1-CO], 566(3)[M+1-HCOOH],
520(4)[566-HCOOH], 492(3)[520-CO],
474(18)[520-HCOOH], 185(100)

TLC: (4:1/ethyl acetate:hexane) R_f=0.61

¹H NMR: δ 0.770(s, 3H, C18-CH₃), 0.892(d, J=6.3 Hz, 3H, C21-CH₃), 0.949(s, 3H, C19-CH₃), 4.720(m, 1H, C3-H), 5.074(q, J=3 Hz, 1H, C7-H), 5.283(t, J=3 Hz, 1H, C12-H), 7.366(s, 1H, amide NH), 7.619(d, J=7.5 Hz, 2H(part of AB), aromatic H), 8.023(s, 1H, C3-formate H) 8.061(d, J=7.5 Hz, 2H(part of AB), aromatic H), 8.106(s, 1H, C7-formate H), 8.168(s, 1H, C12-formate H), 1.059 to 2.482(overlapping m, 25 remaining protons)
(CDCl₃, 500 MHz)

¹³C NMR: δ(C#) 12.23(18), 17.69(21), 22.37(19), 22.83(15), 25.63(11), 26.63(2), 27.28(16), 28.62(9), 29.74, 31.06, 31.39, 34.33, 34.50, 34.57, 34.87, 37.79(8), 40.85(5), 43.02(14), 45.12(13), 47.41(17), 70.75(7), 73.79(3), 75.37(12), 118.77, 124.63, 131.58, 142.79, 160.60(formates), 170.26, 171.65 (CDCl₃, 500 MHz)

Synthesis of Cholic Acid-DHPTA Tetramethyl Ester (22)

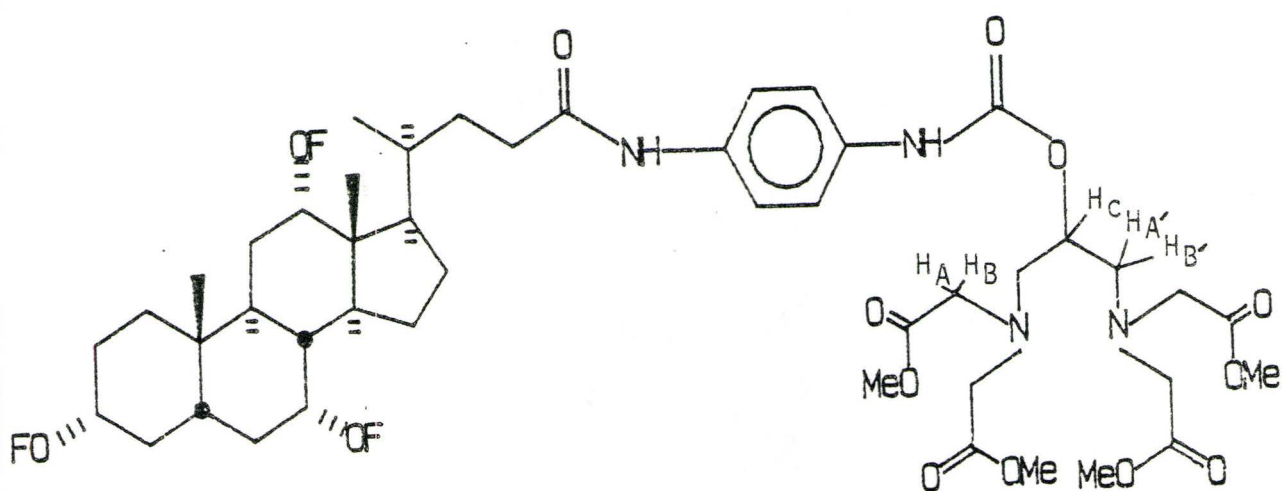
To 1 ml of acetone is added triethylamine (0.055ml, 0.392 mmole) followed by 21 (200 mg, 0.327 mmole). Reverse addition of 21 and triethylamine results in irreversible crystallization of 21. The solution was cooled to 0°C and ethyl chloroformate (0.038 ml, 0.0392 mmole) was added dropwise. After 30 minutes, sodium azide (42 mg, 0.654 mmole) in 0.15 ml H₂O was added and stirring was continued for a further 30 minutes. The solution is poured into 5 ml of ice water and extracted with chloroform (3 x 10 ml). The organic layers were combined, dried with sodium sulfate, and concentrated on a rotary evaporator to yield the acid azide. Dioxane (5 ml) was added and evaporated to azeotrope water. A further 2 ml dry dioxane was added and the solution heated at 85°C for 30 minutes under nitrogen atmosphere. Compound 18 (0.148 mg, 0.392 mmole) in 1 ml dry dioxane was added and heating continued for 30 minutes. The reaction mixture was cooled to room temperature, concentrated on a rotary evaporator and flash chromatographed on silica (4:1 / ethyl acetate:hexane) to produce 192 mg (59.5%) of 22 (semi solid).

MS(FAB+): m/z (RI%); 987(100)[M⁺], 959(54)[M-CO], 915(87),
887(34)[915-CO], 843(22), 815(12)[843-CO], 481(100),
379(100), 277(100)

TLC: (4:1 / ethyl acetate:hexane) R_f=0.57

^1H NMR: δ 0.766(s, 3H, C18-CH₃), 0.887(d, J=6.3 Hz, 3H, 21-CH₃), 0.948(s, 3H, C19-CH₃), 2.990(dd, J_{AB}=14 Hz, J_{AX}=6 Hz, 2H, H_{A'}), 3.046(dd, J_{AB}=14 Hz, J_{AX}=5 Hz, 2H, H_{B'}), 3.592(d, J=17.6 Hz, 4H(part of AB), H_A), 3.627(d, J=17.6 Hz, 4H(part of AB), H_B), 3.678(s, 12H, methyl esters), 3.715(m, 1H, H_C), 4.719(m, 1H, C3-H), 4.908(s, 1H, carbamate NH), 5.073(unresolved q, 1H, C7-H), 5.279(unresolved t, 1H, C12-H), 7.047(s, 1H, amide NH), 7.426(d, J_{AB}=8.5 Hz, 2H, aromatic H), 7.323(d, J_{AB}=8.5 Hz, 2H, aromatic H), 8.021(s, 1H, C3-formate), 8.104(s, 1H, C7-formate H), 8.163(s, 1H, C12-formate H), 1.057 to 2.414(overlapping m, 25 remaining protons)
(CDCl₃, 500 MHz)

^{13}C NMR: δ (C#) 12.24(18), 17.69(21), 22.38(19), 22.84(15), 25.61(11), 26.64(2), 27.27(16), 28.61(9), 31.26, 31.39, 34.35, 34.41, 34.51, 34.57, 34.91, 37.80(8), 40.86(5), 43.02(14), 45.11(13), 47.42(17), 51.56, 55.65, 55.96, 70.74(7), 72.71, 73.77(3), 75.37(12), 119.38, 120.62, 133.55, 134.27, 152.94(35), 160.58(25,26,27), 171.16, 171.92 (CDCl₃, 500 MHz)



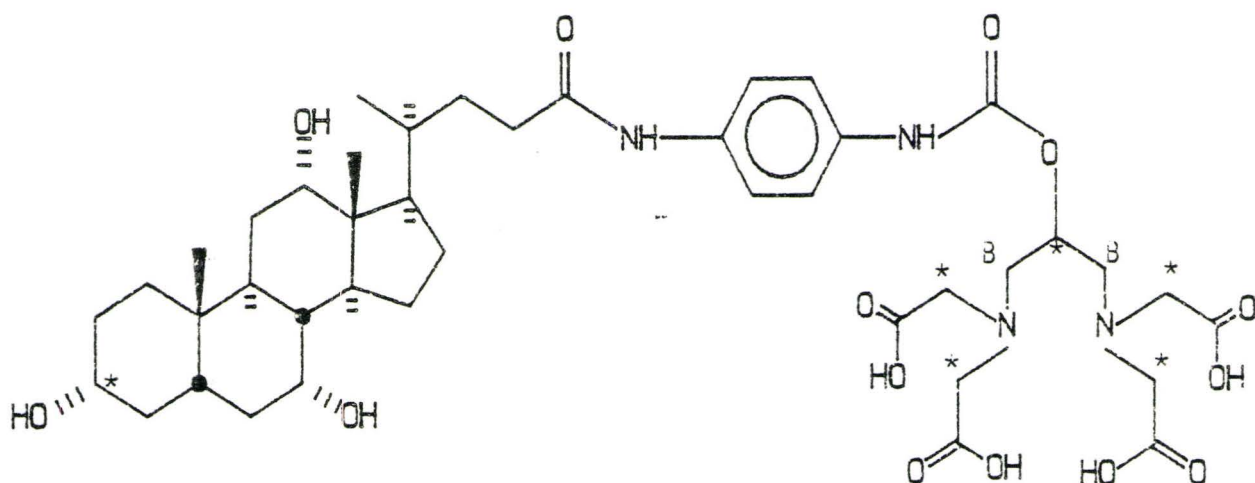
Synthesis of Cholic Acid-DHPTA (9)

Compound 16 (50 mg, 0.051 mmole) was added to a solution of ethanol (3 ml) and 0.5 M lithium hydroxide (8 ml) and the solution was allowed to stir for 2 hrs at 25°C. 1 M HCl was added to adjust the pH to 1 and the solvent was evaporated under reduced pressure. The residue was flash chromatographed on silica (50:50 /ethanol: methanol) to produce 8 (26.7 mg, 62 %).

MS(FAB+): m/z (RI%); 847(94)[M+1], 789(100)[M+1-CH₂COO],
731(35)[M+1-2 CH₂COO's], 673(11)[M+1-3 CH₂COO's],
549(92), 499(100), 445(23), 283(100)

TLC: (50:50 / ethanol:methanol) R_F=0.5

$^1\text{H NMR}$: δ 0.724(s, 3H, C18-CH₃), 0.922(s, 3H, C19-CH₃), 1.083(d, J=6.5 Hz, 3H, C21-CH₃), 3.657(unresolved q, 1H, C7-H), 3.974(unresolved t, 1H, C12-H), 3.517 to 3.664(overlapping m, 9H, HA), 7.328(unresolved d, 2H(part of AB), aromatic H), 7.421(unresolved d, 2H(part of AB), aromatic H), 0.862 to 2.448(overlapping 34 remaining protons), approx. 3.3(m, 4H, HB, obscured by MeOH signal) (MeOH-d₄, 500 MHz)



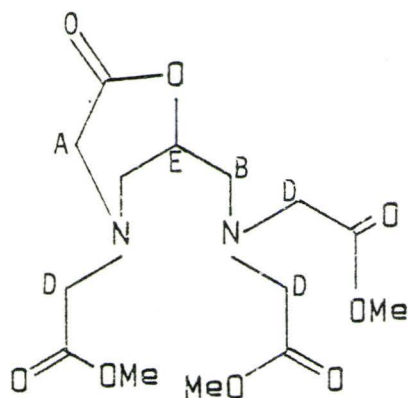
Attempt to Prepare Tetramethyl-1,3-Diaminopropane-Tetra Acetate-2-Tosylate

Compound 18 (200 mg, 0.529 mmole) was dissolved in 5 ml of dry pyridine under an inert atmosphere and cooled to 0°C. Toluene sulphonyl chloride (111 mg, 1.1 equiv) was

added and the solution left to stand at 0°C for 40 hrs. The pyridine was removed under reduced pressure and replaced by 20 ml of chloroform. This was extracted with: NaHCO₃ (2 times 10 ml), 1 M HCl (2 times 10 ml), and water (10 ml). The organic layer was dried with sodium sulfate and concentrated on a rotary evaporator to produce an amber oil: lactone 37 in approx. 80 % yield by ¹H NMR. Little or no tosylate was formed.

MS(EI): m/z (RI%); 379(30)[M⁺], 347(100)

¹H NMR: δ 2.97(d, J=4.5 Hz, 2H, H_A), 3.45(d, J=6 Hz, 2H, H_B), 3.27(s, 2H, H_C) 3.51(s, 6H, H_D), 3.65(s, 9H, -COOCH₃), 4.57(m, 1H, H_E)



Attempt to Couple 19 and 18 Through an Ester Linkage

To a solution of 19 (100 mg, 0.203 mmole) in 10 ml of dry THF at -20°C and under an inert atmosphere was added triethylamine (0.056 ml, 2 equiv) followed by methane sulphonyl chloride (0.018 ml, 1.1 equiv) and the solution was allowed to stir for 30 min. Compound 18 (153.3 mg, 2 equiv) was added and stirring continued for 1 hr at -20°C and 4 hrs at 25°C. No reaction was apparent by ¹H NMR and TLC.

Synthesis of EDTA Tetramethyl ester

EDTA Tetramethyl ester (a colourless oil) was prepared in 88 % yield following the identical procedure as for the preparation of 19.

¹H NMR: δ 2.87(s, 4H, NCH₂CH₂N), 3.63(s, 8H, CH₂'s next to C=O), 3.70(s, 12H, methyl esters) (CDCl₃, 90 MHz) (lit. Hay and Nolan, 1975)

Formation of an Amide linkage - Reaction of EDTA Tetramethyl ester and Octylamine

Two sets of experimental conditions were examined in testing the efficiency of this reaction. First, octylamine (100 mg, 0.770 mmole) was dissolved in EDTA tetramethyl ester (293 mg, 0.770 mmole) and heated at 155°C for 60

min. Small aliquots were retrieved at 15 min intervals and examined by ^1H NMR and TLC. In the second experiment octylamine (100mg, 0.770 mmole) and EDTA tetramethyl ester (293 mg, 0.770 mmole) were dissolved in 0.5 ml of DMSO-d_6 in an NMR tube and heated at 80°C for 40 hrs. ^1H NMR and TLC were performed at various times up to 40 hr. Both experiments showed similar results. Reaction was slow in both cases. An amide NH signal could be detected but the intensity was low even after 40 hrs at 80°C . Significant starting material was also apparent as well as numerous byproducts in both cases. This approach was therefore abandoned.

Attempt to Synthesis 7 from EDTA Tetra Acid

This experiment was performed following the precedent set in a previous synthesis (Hertzberg and Dervan, 1984). EDTA (1 g, 0.0034 mole) was dissolved in 100 ml of dry DMF at 100°C under nitrogen atmosphere. Compound 16 (143 mg, 0.340 mmole) was added and heating continued for 2 hrs. The solution was cooled to room temperature and filtered to remove unreacted EDTA. Significant amounts of unreacted EDTA remained in solution and could not be separated by crystallization due to the similar solubility properties of EDTA and 7. Isolation of the product was therefore very difficult and would require extensive chromatography to be done properly. This approach was

abandoned.

Attempts to Functionalize DTPA at the Central Carboxyl Group
by Various Activation Procedures

Compound 17 (200 mg, 0.559 mmole) was dissolved in 4 ml of dry DMF under an inert atmosphere and reacted:

i) at 0°C with 0.055 ml (1.1 equiv) of oxalyl chloride for 30 min

ii) at -20°C with 0.154 ml (2 equiv) triethylamine and 0.049 ml (1.1 equiv) methane sulfonyl chloride.

iii) at 25°C for 24 hrs with 0.127 g (1.1 equiv) dicyclohexylcarbodiimide and 0.086 g (1.1 equiv) of para-nitrophenol followed by addition of water (1 ml) and heating for 30 min at 75°C

iv) at 0°C for 1 hr until bubbling subsided with 0.99 g (1.1 equiv) carbonyldiimidazole

v) at 80°C for 4 hrs with 0.085 ml (1.1 equiv) triethylamine and 0.071 ml (2 equiv) of chloroacetonitrile followed by addition of water (1 ml) and continued heating for 30 min.

Both reaction mixtures i) and ii) turned dark brown and precipitated tar like substances on the bottom of the flask. This was suspected polymeric material from side reactions with the solvent. Reaction iii) appeared not to proceed at all by TLC (no incorporation of aromatic substituents on TLC). Reaction iv) appeared to proceed

nicely but further trial reactions of the activated species with butylamine at 0°C and -45°C showed comparable reaction rates of the anhydride and acyl imidizolide leading to an inseparable mixture of products. Reaction v) showed some incorporation of the cyanomethyl ester. This was tested by reaction of the activated species with butyl amine. ¹H NMR showed only 20 to 30 % amide formation and so this method was not considered. All trials were deemed unsatisfactory.

Attempt to Prepare DTPA Monoanhydride from DTPA Dianhydride

Compound 17 (300 mg, 0.840 mmole) was dissolved with slight warming in 3 ml dry DMF under an inert atmosphere. The solution was heated to 72°C and water (0.015 ml, 0.840 mmole) was added. Heating was continued for 2 hrs and the solution cooled first to room temperature and then to 0°C. No precipitate was formed as in the case of EDTA monoanhydride formation.

The same reaction was carried out for 20 hrs at room temperature. No precipitate was formed.

The same reaction was performed for 2 hrs at 72°C but using 1.5 ml DMF and 1.5 ml dioxane as the solvent. No precipitate was formed. The monoanhydride approach was abandoned.

Synthesis of the 1,3-Diaminopropane Amide of Cholic Acid
Triformate (26)

Compound 19 (200 mg, 0.406 mmole) was dissolved in 8 ml of dry benzene under an argon atmosphere. The solution was cooled to 5°C and oxalyl chloride (1.5 ml, large excess) was added via syringe dropwise. When addition was complete the solution was allowed to warm to room temperature and stirring was continued for 1 hr. The solvent was removed under reduced pressure and the acid chloride was put under high vacuum to ensure removal of excess oxalyl chloride. The acid chloride was dissolved in 20 ml of dry THF and added dropwise to a solution of 1,3-diaminopropane in 20 ml THF at -78°C. After addition, the solution was allowed to warm to room temperature and let stand for 1 hr. The solvent was removed on a rotary evaporator. TLC showed large amounts of byproduct (reaction at both ends of the diamine to produce 27). Extraction was not possible as the final product could not be extracted into an aqueous layer. Chromatography would be required but was not performed.

Synthesis of 26 using Monotriptyl Protected 1,3-Diamino
Propane

(Monotriptyl 1,3-diaminopropane)

Triptyl chloride (300 mg, 1.07 mmole) was dissolved in 10 ml dry chloroform under an inert atmosphere and cooled

to 0°C. 1,3-diaminopropane (0.6 ml, 9 fold excess) was added. The solution was allowed to warm to 25°C and left to react for 5 hrs. It was transferred to a separatory funnel and extracted (2 times 10 ml) with 5% sodium bicarbonate and then water (10 ml). The organic layer was dried with sodium sulfate and evaporated under reduced pressure to give a clear colourless oil (quantitative).

(Coupling Step)

The acid chloride of 19 (200 mg, 0.406 mmole) was made as previously described. It was dissolved in dry THF (10 ml) and added to a solution of monotrityl diaminopropane (128 mg, 0.406 mmole) and triethylamine (0.056 ml, 0.406 mmole) in dry THF (10 ml). After 1 hr the solvent was evaporated to give a cream coloured solid (28).

(Hydrogenolysis)

The crude product was dissolved in 25 ml of absolute ethanol containing 5% palladium on charcoal (100 mg). The solution was hydrogenated at 1 atmosphere for 8 hrs with no reaction.

Attempt to Couple 18 and 19 via a Carbamate Linkage

Compound 19 (424 mg, 0.860 mmole) was dissolved in 2 ml acetone and cooled to 0°C. Triethylamine (0.138 ml, 1 mmole) and ethyl chloroformate (0.105 ml, 1 mmole) were

added and the mixture was stirred at 0°C for 0.5 hrs. Sodium azide (85 mg, 1.3 mmole) in 0.3 ml of water was added and stirring continued for 1 hr at 0°C. The mixture was poured into 5 ml ice water and extracted with chloroform (3 times 10 ml). The organic layers were combined, dried with sodium sulphate and concentrated on a rotary evaporator at 25°C. Toluene (10 ml) was added and the volume reduced to 5 ml to azeotrope residual water and then heated at 85°C for 30 min to form the isocyanate. Compound 18 (600 mg, 2 equiv) was added and heating continued. The reaction was monitored by TLC up to 48 hrs with no reaction.

In a second experiment the isocyanate was produced under identical conditions. Compound 18 (330 mg, 1.1 equiv) was added followed by dibutyl tin dichloride (261 mg, 1 equiv) as a catalyst. The reaction was heated at 85°C for 24 hrs. The solvent was removed and a portion of the residue was flash chromatographed on silica (4:1/ethyl acetate:hexane). The major reaction product was recovered and was found to be the methyl carbamate (36).

Synthesis of Cholic Acid Triacetate (10)

This procedure is a modification of one reported earlier (Fieser and Rajagopalan, 1954). Under dry conditions and inert atmosphere cholic acid (3.01 g, 0.0073 mole) was dissolved in a mixture of benzene (150 ml), pyridine (37 ml) and acetic anhydride (37 ml). Dimethylaminopyridine (DMAP)

(180 mg, approx. 20% molar equiv.) was added and the solution was stirred for 3 days at 22°C. The reaction mixture was transferred to a separatory funnel and washed with water acidified to pH=3 (5 times 50 ml). The organic layer was evaporated under reduced pressure to produce 2.7 g (69%) of a white foam (one spot on TLC, mp. 112 to 115°C).

MS(CI-NH₃): m/z (R1%); 552(34)[M+NH₄⁺], 534(5)([M⁺]
414(100)[M⁺-2 AcOHs], 354(100)[M⁺-3 AcOHs],
313(22)[414-(CH₂)₄COOH] 300(10), 253(57), 199(9)

TLC: (2:2:1/ethyl acetate:hexane:methanol) R_f=0.59

¹H NMR: δ 0.735(s, 3H, C18-CH₃), 0.829(d, J=6.2 Hz, 3H, C21-CH₃), 0.919(s, 3H, C19-CH₃), 2.133(s, 3H, acetate CH₃), 2.085(s, 3H, acetate CH₃), 2.043(s, 3H, acetate CH₃), 4.576(m, 1H, C3-H), 4.909(q, J=2.75 Hz, 1H, C7-H), 5.089(t, J=2.75 Hz, 1H, C12-H), 0.907 to 2.415(overlapping 25 remaining protons) (CDCl₃, 250 MHz)

¹³C NMR: ppm(C#); 11.82(18), 17.15(21), 21.05(acetate CH₃'s), 22.15(19), 25.21, 26.57, 28.53, 30.28, 30.80, 33.99, 34.18, 37.37, 40.55, 43.02, 44.71, 46.93, 70.40(7), 73.78(3), 75.08(12), 170.23(acetate carbonyls), 178.52(24) (CDCl₃, 250 MHz) (not all C-13 signals individually resolved)

Synthesis of ϵ -N-(α -N butoxycarbonyl lysine)Cholanamide-3,7,12-Triacetate (11)

Compound 10 (0.522 g, 0.970 mmole) was introduced to a dry reaction vessel under an inert atmosphere. Dry chloroform (20 ml) was added and the solution cooled to -20°C using a dry ice / carbon tetrachloride bath. Triethylamine (0.135 ml, 0.970 mmole), was added followed by isobutyl chloroformate (0.133 ml, 0.970 mmole). The reaction was stirred for 2 hrs at -20°C and α -N butoxy-carbonyl lysine (0.241 g, 0.970 mmole) was added and stirring continued at -20°C for 1 hr and then over night at room temperature. The reaction mixture was extracted with 1M HCl (2 times 10 ml) and water (2 times 10 ml). The organic layer was evaporated and the resulting solid was recrystallized from hexane/ethyl acetate to produce colourless crystals (0.42 g, 57%, mp. $69-72^{\circ}\text{C}$).

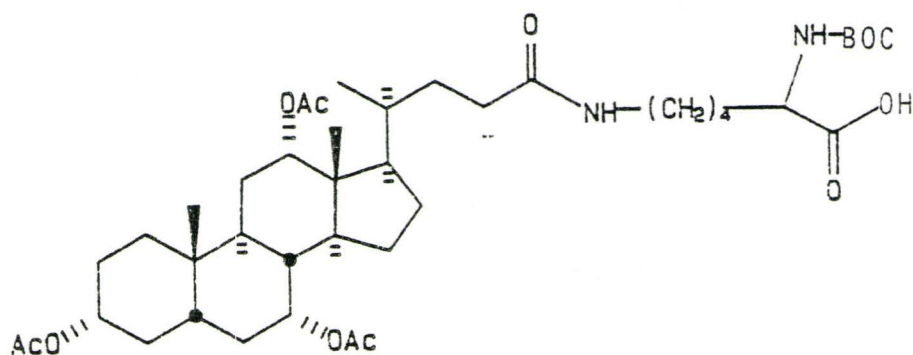
MS(FAB+): m/z (RI%); 763(0.4)[M⁺], 739(0.4), 663(1), 493(6), 339(7), 247(100)

TLC: (2:2:1/ethyl acetate:hexane:methanol) R_f=0.18

¹H NMR: δ 0.772(s, 3H, C18-CH₃), 0.897(d, J=5.9 Hz, 3H, C21-CH₃), 0.921(s, 3H, C19-CH₃), 1.407(s, 9H, t-butyl group), 2.125(s, 3H, acetate CH₃), 2.082(s, 3H, acetate CH₃), 2.044(s, 3H, acetate CH₃), 5.082(m, 1H, C3-H) 4.901(unresolved q, 1H, C7-H), 4.576(unresolved t, 1H, C12-H) 3.161(m, 2H, amide

NH-CH₂-), 3.971(m, 1H, CH α to COOH), 5.692(broad s, 1H, urethane NH), 6.486(broad s, 1H, amide NH), 0.915 to 2.450(overlapping 30 remaining protons) (CDCl₃, 250 MHz)

¹³C NMR: ppm(C#); 12.31(18), 17.68(21), 21.47(acetate CH₃'s), 22.60(19), 22.87, 25.65, 27.21, 28.48, 28.96, 31.35, 33.66, 34.44, 34.76, 37.88, 41.04, 43.46, 45.18, 70.81(7), 74.16(3), 75.55(12), 79.36, 156.17(urethane C), 170.55(acetate carbonyls), 173.81(carboxyl C), 178.10(24) (CDCl₃, 80 MHz) (not all C-13 signals individually resolved)



Synthesis of ϵ -N-(ethanol(α -N-butoxycarbonyl)lysine)Cholanamide-3,7,12-triacetate (12)

Using dry conditions with nitrogen atmosphere 11 (300 mg, 0.372 mmole) was dissolved in 15 ml of dry THF. The solution was cooled to -20°C and triethylamine (0.053 ml, 1 equiv) and isobutylchloroformate (0.049 ml, 1 equiv) were added. The solution was stirred for 15 minutes and ethanolamine (0.024 ml, 1 equiv) was added. Stirring was continued for 1 hr at -20°C then allowed to warm to room temperature. The solvent was removed on a rotary evaporator and replaced with chloroform. The solution was extracted with 5% NaHCO_3 (15 ml), 1M HCl (15 ml) and water (15 ml). The organic layer was dried with sodium sulphate and evaporated to produce 285 mg (91%) of a white foam (1 spot TLC).

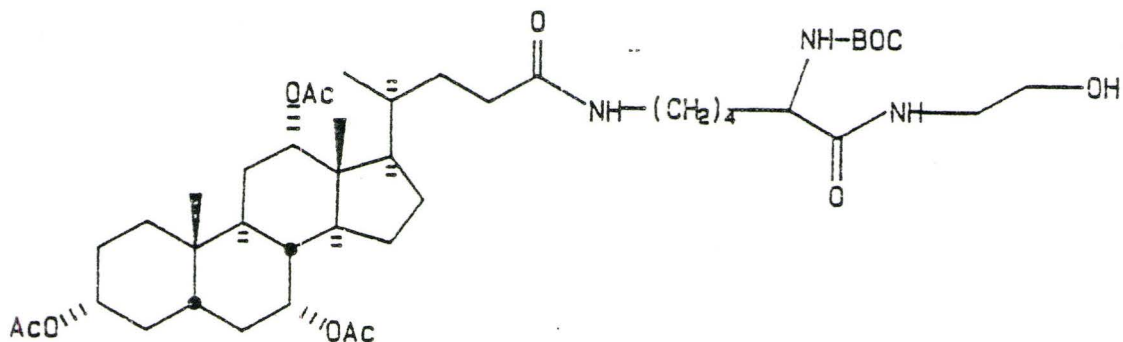
MS(FAB+) m/z (RI%); 807(100)[M+1], 791(24), 764(15),
746(13), 720(10), 706(100), 253(100)

TLC: (2:2:1/ethyl acetate:hexane:methanol) $R_f=0.47$

$^1\text{H NMR}$: δ 0.729(s, 3H, C18- CH_3), 0.818(d, $J=6.2$ Hz, 3H, C21- CH_3), 0.917(s, 3H, C19- CH_3), 1.438(s, 9H, t-butyl CH_3 's), 2.048(s, 3H, acetate CH_3), 2.088(s, 3H, acetate CH_3), 2.138(s, 3H, acetate CH_3), 3.235(m, 2H, H_D), 3.523(m, 2H, H_B), 3.700(t, $J=5$ Hz, 2H, H_A), 4.033(m, 1H, H_C), 4.577(m, 1H, C3-H), 4.907(q, $J=4.5$ Hz, 1H, C7-H), 5.091(unresolved t,

1H, C12-H), 5.294(d, J=7.5 Hz, 1H, urethane NH,
5.772(t, J=6.5 Hz, 1H, amide NH), 6.775(t, J=7 Hz,
1H, amide NH) (CDCl₃, 250 MHz)

¹³C NMR: ppm(C#); 12.61(18), 17.63(21), 21.51(acetate
CH₃'s), 22.48(19), 25.63, 27.17, 28.32, 28.94,
31.55, 31.93, 33.65, 34.37, 34.63, 34.80, 37.74,
38.69, 40.94, 42.31, 43.39, 45.10, 47.54, 54.41,
61.76, 70.70(7), 74.08(3), 75.41(12), 156.32(31),
170.36 and 170.52(acetates), 172.81(30), 173.92(24)
(CDCl₃, 250 MHz) (not all C-13 signals resolved)



Attempt to Reduce Amides using Borane Dimethyl Sulphide Complex

Removal of BOC Group and replacement with Trityl Group

Compound 12 (200mg, 0.25 mmole) was dissolved in dry chloroform (20 ml) under an inert atmosphere and cooled to 0°C in an ice bath. Dry HCl gas was bubbled through the solution for 15 min. Stirring was continued for an additional 1 hr at 0°C and the solution was allowed to warm to room temperature. The chloroform was removed under vacuum on a rotary evaporator and the hydrochloride salt was dried under high vacuum. The hydrochloride salt was suspended in chloroform (15 ml) and triethylamine (0.103 ml, 3 equiv) was added followed by trityl chloride (139 mg, 2 equiv). The solution was stirred for 6 hrs at room temperature and transferred to a separatory funnel. It was extracted with 5% sodium bicarbonate (2 times 10 ml), 1M hydrochloric acid (2 times 10 ml) and water (10 ml). The organic layer was dried with sodium sulphate and the solvent removed on a rotary evaporator to produce 24 which was dried under high vacuum.

Removal of Acetates using Lithium Aluminum Hydride

Lithium aluminum hydride (72 mg, 1.9 mmole, 2.5 mole/mole of acetate) was suspended in dry THF (15 ml) and cooled to 0°C under an argon atmosphere. Compound 24 (crude product from previous step) was added and the solution

stirred for 1 hr at 0°C. The ice bath was removed and the solution refluxed for 1 hr. Methanol (1 ml) was added followed by water (1 ml). The solution was filtered and washed with cold THF. The product (25) was dried under high vacuum.

Borane Reduction

To 100 mg of crude product 25 (max. 0.25 mmole) was added 5 ml of dry THF under an inert atmosphere. Borane-dimethyl sulphide complex (2M)(1.25 ml, 10 equiv) in 5 ml THF was mixed with 24 at 0°C in a round bottom flask equipped with reflux condenser. The solution was stirred for 15 min at 0°C and refluxed for 3 hrs. The mixture was cooled to room temperature and 0.5 M HCl (10 ml) was added. The solution was evaporated on a rotary evaporator and the product analyzed by TLC. The product contained a mixture of compounds and no purification was attempted.

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