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SYNTHESIS OF SPORIDESMIN BASED ANTIGENS

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ABSTRACT

In a program aimed at developing a chemically derived vaccine against sporidesmin-A, the toxin which causes the pasture disease facial eczema, two haptens have been prepared, coupled to protein carriers, and tested for immunogenicity.

The hapten, 2-amino-5-chloro-3,4-dimethoxybenzyl alcohol has been synthesised from vanillin and the general synthetic methods developed in the course of this work have been used to prepare a large number of related vanillin derivatives for use in cross-hapten studies. The carbon-13 nuclear magnetic resonance spectra of 16 vanillin derivatives have been obtained, and two independent methods of deducing vanillin substitution patterns have been developed.

5-Chloro-6,7-dimethoxy-N-methyl-1H-indole has been prepared by a new route and its 3-oxoacetic acid derivative has been synthesised for use as a hapten. The Vilsmeier-Haack intermediate, 1-methyl-3-(N,N-dimethylimonio)methyl-1H-indole chloride was isolated and a number of possible synthetic routes from it to pyrroloindoles were explored.

The vanillin and indole haptens have been coupled to protein carriers to form antigens which generated a low antibody response. However, the use of *Mycobacterium phlei* as a carrier greatly increased the antibody response but the antibodies produced did not interact strongly with sporidesmin-A.

Part IIntroductionSection AHistory of the Disease, Facial Eczema

Facial eczema, a major disease of ruminants in New Zealand, occurs in the autumn when warm rains fall on dry pastures, and is so named because of the serious facial lesions it causes in sheep and cattle. Moreover it results in severe damage to livers and bile ducts, often causes death, and those animals that do recover are unthrifty, with poor lambing and calving yields in the following spring. It ranks as a major economic loss in the agricultural industry of New Zealand.

Although the disease was described by Gilruth as early as 1908¹, concerted scientific research did not begin until 1938. Characterisation of the cause proved exceptionally difficult², but in 1958, soil specialists of the New Zealand Department of Scientific and Industrial Research identified it as one of eighty species of fungus found in toxic pastures. The toxic fungus was originally identified as *Sporidesmium bakerii* but this proved to be incorrect and it was later re-classified as *Pithomyces chartarum*. A toxin had been suspected as the causative agent for some time, but isolation was difficult because of its low and erratic occurrence, its chemical fragility, and the difficulties in testing for its presence. The toxin was found to reside in the spores, and the isolation of a high sporing strain of the fungus greatly simplified purification. A combination of delicate concentration techniques and a series of chromatographic separations allowed Synge and White to isolate the pure toxin in 1959.³ It was named after the original fungus classification as "sporidesmin".

Figure 1.1, Sporidesmin-A

Elucidation of the structure of the toxin was also difficult. Elemental analysis was complicated by the co-crystallizing of approximately one mole of solvent, and degradation gave no recognisable products. The structure and absolute configuration were finally determined by crystallographic means.^{5,6,7}

The compound has a pyrrolo-indole fused to a dioxopiperazine ring, with a cage formed by a bridging disulphide group. Biological activity is associated with the disulphide cage functionality. A number of related compounds have now been discovered, and their structures are given in section B. They differ in the sulphur functionality or in hydroxylation at carbon-11. (For nomenclature, see footnote).

Footnote: Up to volume 67, Chemical Abstracts lists the group under sporidesmin, and from volume 68 it is listed as 3,11a-Epidithio-11aH-pyrazino[1',2':1,5]pyrrolo[2,3-b]indole-1,4-dione, with appropriate substitution, and as sporidesmin in the keyword index. Numbering is based on:

1.2

In this thesis, "sporidesmins" will refer to the whole group, and "sporidesmin", without a following letter (A-G), will refer to unspecified mixtures as in toxicological studies using native extract.

The mechanism of toxic action is not clear, although it has been proposed that the disulphide cage may form dithio links to cysteine residues on a liver cell surface.⁸ Attack certainly occurs on the liver cells, with an increase in permeability resulting in the loss of NAD coenzymes,⁹ and then cell necrosis. Pathological examination shows the liver and bile ducts are attacked first, and the subsequent liver damage and bile occlusion prevents the elimination from the blood of a chlorophyll metabolite, phylloerythrin.¹⁰ This porphyrin circulates in the blood to areas of skin exposed to sunlight, where it absorbs and transfers the energy in some unknown manner to the nearby cells, causing severe damage. All unprotected skin is sensitive, which in sheep is primarily the face. The damage results in serious blistering, serous exudation and necrosis until the facial area is a mass of sores. Thus although the liver is the primary area of damage, the disease is called facial eczema after the more obvious effects on an animal's face.

It is noteworthy that affected animals avoid sunlight, and stock losses can be halved by the provision of shade. There is no known cure, however, and the economic loss has prompted farmers to demand effective counter measures. An early method was a district facial eczema radio warning system, based on weather conditions and Departmental spore counts. Methods have now improved to the point where farmers can make their own spore counts and develop a knowledge of dangerous areas on their farms. This has the enormous psychological advantage of replacing a fear of an invisible enemy with a concept of an observable fungus which can be largely avoided by proper grazing management.

Another preventative measure involves spraying paddocks with a fungicide, but even the most economic of these, thiabendazole (1.3),^{11,12} is rather costly to apply over large acreages, and is most efficiently used on emergency reserve paddocks.

Thiabendazole

1.3

As more and more is learned about the disease, new approaches are tried, but at present, none is really successful. The disease is also more widespread than was first thought. It has been reported in Victoria, Australia,¹³ appears to be present in South Africa, and possibly the northern hemisphere as well. Closely related diseases occur in Nova Scotia¹⁴ and a successful cure will be of international interest.

There has been a recent development of the use of zinc compounds as a preventative measure,⁸ but as they must be given in high doses concurrent with, or immediately prior to sporidesmin intake, no practical treatment is readily apparent. Work is, however, continuing in this field, particularly since the mode of action of zinc-based prevention is unknown. The effect may be due to reversible binding of zinc ions to free thiol groups, which prevents sporidesmin attack on the cell membrane. The use of more sophisticated chemicals is possible, but they must be cheap or else only needed in small quantities. A large difference has been observed in the susceptibility of various animal species to sporidesmin poisoning, but this may arise, not from species specific immunity, but from the toxicity being a function of liver-cell surface area, rather than of body weight. Dosages based on body weight would give a disproportionate amount of toxin to small animals compared to larger animals, such as a horse or cow.

Within a species, there is also considerable difference in susceptibility. If this difference can be shown to be genetically

determined, then possibly the best long term solution to the problem is a breeding program. This would take about twenty years to complete, and would have the disadvantage of committing farmers to a single strain of sheep. This same disadvantage would occur in cattle, and in any other species likely to be grazed such as goats and horses. Economically it may be justifiable, but a more general solution would seem preferable.

The object of the research described in this thesis has been to make a vaccine capable of preventing the disease in any species. Originally, a vaccine was considered inapplicable¹⁶ due to the smallness of the sporidesmin molecule and its lack of affinity for proteins. These reasons are no longer thought to be insurmountable and the methodology to achieve vaccination will be detailed in section D.

Section B

The Epipolythiodioxopiperazine Class of Compounds

The chemistry of the dioxopiperazines, especially those with sulphur bridging, has been reviewed recently by Taylor^{14,16} and by Sammes.¹⁷ These reviews briefly discuss the sporidesmins, whose detailed chemistry is largely covered by the series, Sporidesmins I-XV.^{4,18-31} A chart, summarising the reactions, is given by Taylor,¹⁶ and almost all the sporidesmins have been synthesised from sporidesmin-A (1.1). The authors of the series made no attempt at a complete synthesis of sporidesmin-A, but some degradation products were prepared.²⁰ This section will briefly cover only those reactions relevant to the research described in the main body of this thesis, together with an outline of the chemistry of the class of compounds to which the sporidesmins belong.

The structure of sporidesmin-A (1.1) was established by X-ray diffraction crystallography, and all chemical and spectroscopic evidence is in agreement with this structure. Further purification of crude sporidesmin revealed the presence of a family of compounds, and seven have been isolated and characterised. The designation sporidesmin-A, sporidesmin-B etc., distinguishes them, but if no letter is used, sporidesmin-A is intended in the early literature, and in toxicological work, "sporidesmin" may refer to the unpurified extract containing a number of sporidesmins. All the sporidesmins are first reported in the series of papers, "Sporidesmins", except for sporidesmin-A³ and sporidesmin-C.³² (Sporidesmin-B,^{4,18} -D,²⁴ -E,²⁵ F²⁴ and sporidesmin-G,²⁸). Sporidesmin-G has also been structurally analysed by X-ray crystallography.³³ Taylor¹⁴ mentions a sporidesmin-H, but no reference to its isolation is given. The General Subject Index of

Chemical Abstracts, under "Nomenclature, new natural products", lists sporidesmin-G in volume 76 (isolation) and volume 80 (crystal structure), but no listing of sporidesmin-H is made under that heading in any volume. The supplement to the Index Guide, volumes 77-83, lists all the sporidesmins and their IUPAC names, except for sporidesmin-H. It is doubtful, therefore, if the isolation and characterization of sporidesmin-H has been published. No further reference to this compound will be made.

- 1.1, Sporidesmin-A; R=OH, x=2
- 1.4, Sporidesmin-B; R=H, x=2
- 1.5, Sporidesmin-C; R=OH, see figure
- 1.6, Sporidesmin-D; R=OH, see figure
- 1.7, Sporidesmin-E; R=OH, x=3
- 1.8, Sporidesmin-F; R=OH, see figure
- 1.9, Sporidesmin-G; R=OH, x=4

1.5

1.6

1.8

The sporidesmins (1.1, 1.4-1.9) fall into two groups, the biologically active group whose dioxopiperazine group is bridged by sulphur atoms of varying number, and an inactive group (-C, -D, -F) in which the bridge is broken or methylene linked. Sporidesmin-E (trisulphide) is about ten times as toxic as sporidesmin-A (disulphide) or sporidesmin-G (tetrasulphide), and the others are less toxic than these.¹⁴ It is not known what is the toxic component *in vivo*, as the

number of atoms in the sulphur bridge may depend upon the metabolism of the test animal. The disulphide-strain release theory of poisoning would predict the disulphide to be more toxic, and can be regarded as very tentative indeed.

1.10 Gliotoxin

1.11 Aranotin; R=H

1.12 Acetylananotin, R=COCH₃

1.13 Apoaranotin

1.14 Verticillin-A ($R^1=R^4=H, R^2=Me, R^3=OH$)1.15 Chaetocin ($R^1=R^3=R^4=H, R^2=CH_2OH$)

The toxic behaviour observed for sporidesmins is similar to that reported for other epipolythiodioxopiperazines. Gliotoxin (1.10) is another well studied member of the group, its structure being first deduced correctly by R. B. Woodward and co-workers.³⁴ Others in the

group are aranotin (1.11), apoaranotin (1.13) and their dimethyl dithio derivatives, and two compounds which are dimeric sporidesmins, verticillin-A (1.14) and chaetocin (1.15).^{14,17} There are strong similarities between members of the group implying a similar biosynthetic pathway, and only recently has a compound been discovered which has the opposite cage chirality to sporidesmin-A.³⁵ The similarities are neatly linked by the epoxide mechanism suggested by Neuss *et al.*,³⁶ and both Taylor and Sammes support it (Scheme 1a).

Scheme 1a

The corresponding sporidesmin mechanism is: (Scheme 1b)

Scheme 1b

Once the pyrrole ring is formed, then the amine and acid substituents of 1.17 are restricted to conformations aiding dioxopiperazine formation.

Sporidesmin has been established as a biosynthetic condensation product of tryptophan and alanine, by the use of ^{14}C -tracers,^{37,38} and radioactive sulphates have been used to produce (^{35}S)-sporidesmin-A biosynthetically.³⁹ The method could be extended to check whether sporidesmin-C is a condensate of tryptophan with serine or cysteine or else is derived naturally or artificially from sporidesmin-A.

The sporidesmin group can be interconverted to a large degree *in vitro*, which implies the possibility *in vivo*:

- 1) The sulphur bridge can be enlarged by the use of dihydrogen disulphide.²⁶ (Scheme 1c)

Scheme 1c

Sporidesmin-A → Sporidesmin-E and Sporidesmin-G

- 2) The reaction is reversed by triphenylphosphine, and, at a very much slower rate, sulphur can be extruded from the disulphide to give a monothio-bridge derivative.²⁷ Sporidesmin-E can be disproportionated to sporidesmin-A and sporidesmin-G by photolysis.²⁶

If ^{35}S -dihydrogen disulphide is used to make the sporidesmin-E, all the radioactivity is found in the tetrasulphide (-G) after photolysis.

- 3) Sulphur elimination to form a pyrrolo indole can be achieved by the use of anhydrous acids on the di-O-acetate of sporidesmin-A.¹⁹ (Scheme 1d).

Scheme 1d

Dehydrogliotoxin behaves similarly, and the product (1.21) was synthesised as shown in Scheme 1e.²²

- 4) Sporidesmin-D can be synthesised from sporidesmin-A via a dithiol intermediate, which is easily reoxidised to the epidisulphide.²⁵ (Scheme 1f).

The alkylation of the dithiol (1.22) is facile, and Ronaldson³⁰ has also prepared a dithiodiglycolate using an ester of chloroacetic acid.

There is a considerable body of degradative work in the literature. This will not be summarised, but will be referred to in the main part of this thesis when appropriate.

Section C

The Synthesis of Epipolythiodioxopiperazines and Sporidesmin-A

Although facial eczema is primarily a New Zealand problem, there are two factors which have combined to spur international research in this area. Firstly, sporidesmin belongs to the widely distributed class of naturally occurring compounds which were described in the previous section. Secondly, and more importantly, this group has high physiological activity. Simple members of the series can severely inhibit viral RNA synthesis,⁴⁰ and others, such as gliotoxin, are toxic to fungi, bacteria and mammals.¹⁶ Since the mode of toxicity is unknown, but believed to involve membrane attack, the varying properties of members of this class are of interest. Considerable research has gone into their synthesis, and progress has been rapid. This section will describe the various approaches, and the recent total synthesis of sporidesmin-A.

There are two basic approaches to synthesis of the toxin: formation of the dioxopiperazine ring before or else after the polysulphide bridge has been established. Ring closure after polysulphide bridging has yet to be achieved. Rae and coworkers attempted to link two α -thioamino acids prior to ring closure (Scheme 1g), found formation of the monomeric amino acids difficult, but did finally succeed in forming the wanted system.^{41,42,43}

Scheme 1g

However this diacid, 1.24, would not form amide derivatives, and even the monomer, 1.23, was resistant to amide formation. There have been a number of other attempts,¹⁷ but the most successful has been that of Witkop *et al.*,⁴⁴ who formed one of the amide bonds before sulphide inclusion (Scheme 1h). The gliotoxin analogue, 1.25, was obtained, but this monosulphide could not be ring closed, even though monosulphide diketopiperazines are known. This could be because the carboethoxy and methylamino groups are *trans* related.

Scheme 1h

Successful methods have always involved the initial preparation of dioxopiperazines, then substituted with sulphur. Dioxopiperazines may be synthesised in a number of ways,¹⁷ The formation of a symmetrical ring may be achieved simply by refluxing an amino acid in ethylene glycol, but this may result in racemation or else polymer formation.

The use of Leuch's anhydrides (1.26) with aziridine is a new method that gives a very pure product with almost no polymerisation.⁴⁵ (Scheme 1i).

Scheme 1i

The syntheses of unsymmetrical rings use typical peptide synthesis methods to form dipeptides which are then ring closed. (Pyrrolo rings aid ring closure¹⁷ and fused pyrrolo rings are often found in both naturally occurring and synthetic dioxopiperazines). The dioxopiperazine of tryptophan and alanine have been prepared in all four possible stereoisomeric forms by these methods.⁴⁶ (Scheme 1j). Higher optical purity is obtained by refluxing in methanol⁴⁷ rather than ammonia, in step (3).

Z = BnOCO, but Sammes¹⁷ recommends $tBuOCO$, removed by formic acid

Scheme 1j

Bridging the ring with sulphur is done indirectly. The first synthesis of an epidithiodioxopiperazine, 1.28, was reported by Trown⁴⁰ (Scheme 1k) although his method is not general, as dehydrobromination occurs with 3,6 disubstituted dibromodioxopiperazines.¹⁷

Scheme 1k

The acidity of the ring hydrogen atoms of 1.27 is low. Hino and Sato⁴⁸ found that the use of sodium hydride and an electrophile, such as sulphur chloride (S_2Cl_2), on 1.27 or 1.30, resulted in recovery of starting material. They then developed an efficient synthesis of an activated ring (1.29) and found that sulphur chloride substituted across the ring to give a mixture of polysulphide bridges (Scheme 1l)

Scheme 1l

Use of the dipotassium salt analogue rather than the ethyl ester (1.29) permitted facile decarboxylation in the presence of sulphur chloride to give the Trown product (1.28). As in the natural products series,

the sulphur bridge could be opened with sodium borohydride and alkylated.⁴⁹ However, another typical reaction, sulphur extrusion with triphenylphosphine, resulted in the formation of dimers, by way of a postulated 1,4-dipolar intermediate.⁵⁰

The most extensive series of reactions are those reported in the series by Schmidt *et al.*, "On amino acids and peptides", I-XVI.⁵¹⁻⁶⁶ They found that a carbanion could be generated from a non-activated dioxopiperazine (such as 1.30) by the use of amine anions or else sodamide.⁵³ The carbanions reacted with a variety of electrophiles to give sulphur and oxygen derivatives. In the case of diproline-dioxopiperazine (1.29), hydroxyl groups were introduced *trans* to one another, but this product could be racemised to the thermodynamically preferred *cis*-diol by acid. As the *cis*-diol was easily converted to the *cis*-dithiol, this gave rise to an excellent general method,⁵⁷ (Scheme 1m).

Scheme 1m

Use of a semiproline dioxopiperazine proved to give stereoselective hydrogenation of an arylidene derivative - which in the case of tryptophan permits L-amino acid derivatives to be formed.⁵⁸ A variation of the Ugi Reaction⁶⁷ gave derivatives similar to ring opened gliotoxin.^{60,61} From an acyl pyrrole, a five step reaction gave the appropriate dithiodioxopiperazine (Scheme 1n).⁶⁶

This reaction is the last by Schmidt in the series to date, and an extension to a gliotoxin or sporidesmin synthesis would not be difficult, given suitable substrates, that is, an indoline derivative in the gliotoxin case, and a pyrrolo-indole in the case of sporidesmin.

In 1973, some time after the present research was begun, three consecutive publications appeared as communications to the *Journal of the American Chemical Society*. They discussed a general synthetic method of making epidithiodioxopiperazines with two specific examples, the total formal syntheses of sporidesmin and of dehydrogliotoxin.^{68,69,70} The method involved the use of an acid and base stable derivative of the epidisulphides, with stepwise addition of substituents at positions 3 and 6. The protecting group was removed by 1) oxidation then 2) a catalysed rearrangement with boron trifluoride to give the epidisulphide (Scheme 1o)

Scheme 1o

The intermediate (1.31) will form a monocarbanion in tetrahydrofuran at -78°C , and the hydrogen removed by butyllithium is that α to the carbonyl that is adjacent to the group of R. One synthesis given as an example is the preparation of the ($\pm$) serine-alanine derivative (1.32), (Scheme 1p).

Scheme 1p

If groups other than methyl are required as nitrogen substituents, they may be placed there before the protecting system is established, as in the case of the dehydrogliotoxin synthesis,⁶⁹ or else a labile group may be used as in the case of sporidesmin.⁷⁰

The synthesis of sporidesmin was based on the linking of an indole derivative to a dioxopiperazine derivative with a protected sulphur system as above, then oxidative ring closure to give the basic skeleton, (Scheme 1s).

The protected dithiodioxopiperazine moiety was synthesised as follows (Scheme 1q):

The indole moiety (1.38) was made from dimethoxyoxindole (1.35), (Scheme 1r).

Scheme 1r

The syn and anti mixture (1.39) resulting from reacting 1.34 and 1.38, was hydrolysed, then the diastereoisomers of 1.40 were separated.

Reduction and oxidative ring closure gave the diacetate 1.42, (Scheme 1s).

The diacetate (1.42) was found to be identical by IR, UV, NMR, MS and t.l.c. with an authentic sample prepared from sporidesmin-A. No optical rotation was measurable, as the synthetic product is the ($\pm$) racemix of enantiomers. Some of the reactions require further elucidation. Reduction of 1.40 was stereospecific, and, as reduction would not occur when the nitrogen was protected (1.39), an intramolecular hydride shift was considered a crucial step in the reduction. Iodosobenzene diacetate, the reagent used to obtain the diacetate 1.42, can be regarded as a source of $\text{AcO}^\oplus$, and the addition occurs so as to allow the greatest separation possible of the bulky indole and thioacetal residues. The diacetate (1.42) was obtained from sporidesmin by borohydride reduction (Scheme 1f), then protection (as in Scheme 1o, first step) followed by acetylation. Sporidesmin-A (1.1) was regenerated by deacetylation then deprotection (as in Scheme 1o).

Sporidesmin-B (1.4) has also been synthesised,⁷¹ by reducing the acetate (1.41) with sodium cyanoborohydride, then ring closure using benzoyl peroxide in the presence of a radical scavenger. The

reason for the change in oxidant from iodosobenzene diacetate is not given, but may be related to work being simultaneously done by some of the Havard group on tetrodotoxin, the puffer fish toxin.⁷² The three communications are each worthy of a full paper, and many of the above points should be elucidated when the work is fully reported.⁷³

There is a degree of relationship in all the above epipolythiodioxopiperazine work. The introduction of the thioacetate in the sporidesmin synthesis follows largely the method of Trown, and general methods for forming the dithiodioxopiperazines have been developed by Schmidt. The complete synthesis of most members of the sporidesmin family have been formally completed, since they have already been prepared from natural sporidesmin-A. Sporidesmin-C (1.5) may be exceptional, but it is also possible that it is an artefact of acetylation.⁶⁸⁻⁷⁰ Therefore the methods developed by Kishi *et al.*, may be considered the culmination of sporidesmin synthesis work. Interest has now shifted into the toxicology of the compound, and into devising a cure for facial eczema. This thesis, directed towards such a cure, is a combination of compound synthesis and immunological application. The theories governing the work are outlined in the next section.

Section D

An Introduction to Immunology

Immunology is the study of the immune system of vertebrates, a highly versatile and specific defence system against invasion by foreign bodies. In particular, it is the study of a system in which mobile cells and secreted substances, both capable of identifying foreign bodies in the very complex environment of a vertebrate, interact to immobilise and destroy a wide range of invaders, from bacteria and viruses to small proteins such as the diphtheria toxin. The practice of immunology is ancient,⁷⁴ but competent use began with Edward Jenner in 1798, when he noted that the virus of cowpox (vaccina) when inoculated into humans, could also produce lasting protection against smallpox. These "vaccination" methods were later developed by Louis Pasteur, who established the germ theory, to counter such diseases as anthrax and rabies. The Salk vaccine against poliomyelitis is noteworthy in modern times, but surprisingly most of these advances were achieved with a very limited knowledge of how the immune system works. Since 1950, a major expansion of this field has taken place, and discoveries are occurring so rapidly that textbooks are soon dated.

There are four major expressions of immunological activity.^{75a,76}

- 1) Cell mediated immunity, which is a rapidly initiated, short term attack by lymphocyte cells on an antigen (an invader).
- 2) Humoral immunity, which is a more slowly initiated, longer lasting production of agents called antibodies, found in (cell-free) serum.
- 3) Memory, which is the ability of the immune system to respond to repeat attacks by an antigen much more quickly than it did initially.

- 4) Immunological tolerance, which is a weak or null response of the immune system to a normally effective antigen, and is brought about by very high or low doses.

Only the second activity, on antibody response, will be discussed in detail, as it most clearly and simply illustrates the specificity of the immune system, and allows the deduction of the principles guiding the synthetic work described in this thesis. The antibody (defending substance) and the antigen (foreign invader) can both be described in chemical terms, and their interaction as a kinetic reaction involving weak bonds. The description will be biased towards the chemical side, but it should be noted that the variation in structure typical of biological systems precludes absolute characterisation. The definitions in this section will be in terms of the function a substance plays in a living system, and a purely chemical description of the immune system is not possible.

The Antibody. Antibodies belong to the class of compounds known as immunoglobulins. Globulins are proteins low in carbohydrate content, and low in water solubility. The immunoglobulins are defined^{77a} as animal proteins with known antibody activity, or related to such antibodies, and hence possessing specificity for an antigen. There are five classes of immunoglobulins (Ig) possessing full antibody activity, and these antibodies are designated by letters as: Ig G, Ig M, Ig A, Ig D and Ig E.^{78a} They vary in structure from species to species, and even within the individual, but each class is distinct in its functions.

The commonest class, Ig G, makes up 70% of the serum antibodies, and its availability has meant that it has been studied most fully. In addition, a disease called multiple myeloma,^{74b} which is caused by a

single, antibody-producing, plasma cell proliferating out of control results in chemically analysable amounts of immunoglobulin, generally Ig G, subunits. These myeloma proteins were made use of by Porter in deducing the chain structure of antibodies.⁷⁹ By cleaving the molecules with pepsin at one site, papain at another, then by reducing all disulphide links and alkylating them, Porter obtained fragmentation patterns that implied a four chain structure, 1.43 (Scheme 1t). The molecule consists of two identical polypeptide chains of 445 residues called the "heavy" chains, and two "light" chains, also identical, of 215 residues. There is a hinge region where the molecule is flexible, and the heavy and light arms close together to give a pair of binding sites at their tips.

Scheme 1t

The fragmentation was extended by Edelman who used Cyanogen bromide and the above methods to obtain seven fragments of the heavy chain, and three of the light chain.⁸⁰ These were then further fragmented into two sets of oligopeptides by trypsin and chymotrypsin. Sequencing of all these fragments allowed the deduction of the amino acid sequencing of the entire molecule, 1320 residues in all. The amino acid analysis showed that there were six pairs of "domains" in the Ig G molecule (Scheme 1u).

The antigen binding region of the molecule is formed from the N terminals of the light and heavy chains and is known as the variable region, because the amino acid sequencing differs greatly from antibody to antibody. The two domains in the variable region are labelled V_H and V_L (for heavy and light chain respectively). There is also a set of four pairs of sequences in the "constant" region, C_L , C_{H1} , C_{H2} , C_{H3} . Apart from a slight amount of variation that is genetically determined, these domains are constant within a species. The domains can be identified by the moderate degree of amino acid sequence similarity they display. There are internal disulphide bonds maintaining the conformation of the domains, and the major carbohydrate portion of the antibody is sited in domain C_{H2} , (Scheme 1u). The variable regions extend over 110 residues, and comparison of chain sequencing shows hypervariable sections, which seem capable of folding together to form a hypervariable region, that is, the binding site on the end of the molecule.^{78c}

Domain Model

Folding of the four chains in IgG1.
Chains have disulphide links at
cross over or touching positions

• = carbohydrate.

[According to Jerne⁸¹].

Scheme 1u

The other antibody classes have far more carbohydrate than has Ig G, but the significance of this is unknown. The next most abundant class is Ig M, which is a pentamer of five Ig G-like units linked in a ring.^{78d} It is decavalent* in theory, but steric restrictions may lower the valency to as low as five. The other antibodies are divalent monomers like Ig G, although Ig A can occur in dimeric forms.

The Antigen. An antigen is a substance which will produce an observable response by the immune system, specifically directed against the antigen. Not all foreign bodies entering a vertebrate induce antibody activity. One of the limiting factors is size. To be an effective immunogen, that is, a substance capable of initiating antibody response, the

Footnote: Valence refers to combining ratios rather than the more modern electron bond theory.

compound must be rather large. The required size varies for fats, proteins or carbohydrates, and the response does not vary sharply with molecular weight. Generally, a weight of more than 100,000 dalton is effective. Dextrans of 50,000 dalton are poor, and the lower limit in proteins is 5,000 to 10,000 dalton. The reasons for this are poorly understood.

The memory effect has a great bearing on the response to an injection of antigen; a second injection producing both a stronger and more rapid response. For this reason, high antibody levels are produced by giving a series of injections, such as one every two weeks for two months.

Antigens may be regarded as natural, artificial, or synthetic.^{78e} Natural antigens are obtained from natural sources without modification, and although their structure may not be well known, they have been intensely studied because of their use in vaccines. Thus diphtheria toxin, a rather small protein, can be attenuated by treatment with formalin to a much less dangerous form, called a toxoid, which is the basis for a vaccine. The attenuation of a biologically dangerous property, whilst still retaining recognition, is an important general principle. Attenuation occurs because the antibody recognition site is not very large, so that if toxicity is associated with one part of a molecule, and the antibody specific site with another, then in principle it is possible to remove the toxic properties without affecting the ability of the antibody to recognise the antigen. Conversely the antigen may so alter that it can no longer trigger the memory effect, yet still retain virulence, which is the reason why influenza epidemics often occur.

Although an antigen must be large, to be an immunogen, the binding site on the antibody is specific to a small part of the antigen. This feature was first thoroughly exploited by Landsteiner, at about the time he received the Nobel prize for his immunological work identifying the A, B and O blood groups (1930).⁸² Small, well defined molecules were covalently linked to a protein, and then this artificial antigen was used to stimulate antibodies some specific to the carrier protein but, more importantly, other antibodies specific to the small, added molecule.⁸³ In favourable cases, high specificity was obtained, with distinction between the ortho, meta and para isomers of aminobenzene sulphonic acid being possible.^{75b,83} Numerous other examples defining the specificity of reaction now exist.^{84a} The work "haptens" was coined to describe these small molecules, and a hapten is defined as a substance that is incapable by itself of inducing an antibody response, but that is capable of reacting with an antibody in some observable way.

It should be noted that 1) A hapten, whether bound in an exposed position on a carrier, or free in solution will always react with an antibody specific to it. 2) As long as a hapten occupies an antibody binding site, no other hapten can occupy that site. The first note is important since if an antigen can be made with a determining group on it closely resembling sporidesmin-A and antibodies generated against it, then free sporidesmin-A in the blood will be a hapten to which the sporidesmin-specific antibody will bind.

The size of a hapten may vary, for although the original injected hapten will occupy the entirety of the binding site, a later, different hapten somewhat smaller in size might possess sufficient reactivity to bind to the antibody but not occupy the whole site and be fully bonded. The differing reactivities depend on the nature of the bonds. These are

all weak and reversible, and a strong overall binding effect can be obtained by a combination of the following associative forces:^{84b}

Coulombic: Unlike charges attract inversely as the square of the distance between them.

Hydrophobic: A water molecule prefers a polar environment, so non-polar groups aggregate.

Van der Waals: Induced polarisation of two nearby atoms.

Hydrogen bonding: An electron-deficient hydrogen atom is attracted to a non-bonding electron pair.

Dipole Interactions: Attraction resulting from varying electronegativities. An additional contribution to the energy of the reaction (ΔG) is obtained by the entropy increase resulting from the release of bound water molecules with replacement by a single hapten.

These attractive forces are ultimately offset by electron repulsion from orbital overlap, which varies sharply and inversely with distance (to the twelfth power approximately) and this implies a strong correlation between the shape of a hapten and its affinity for a binding site.

Affinity (K) is defined as the equilibrium constant cf. the monovalent reaction, and can be measured by a number of techniques.^{84c}

Ab = Antibody

Ag = Antigen

$$K = \frac{[AbAg]}{[Ab][Ag]}$$

$$\Delta G = -RT \ln K$$

The size of a binding site can be established by comparing the affinities of a given antibody for a number of haptens, and determining when maximum binding occurs, as a function of size. A number of studies have shown the binding cavity will accommodate about 5 or 6 amino acid or glucopyranose residues. As neither the hapten nor the antibody are rigid, the estimate cannot be more precise, but size does not seem to

vary with the antigen. Therefore the ideal free hapten is about five amino acid residues in size, since increasing the hapten will not increase affinity, and a smaller free hapten will lack a complementary set of bonds to part of the antibody. If a molecule smaller than five residues is linked to an antigen, then the determinant group will include the link, and a high affinity hapten will be the small molecule linked to a suitable protein residue.

The use of synthetic antigens - linear and branched polymers and copolymers of amino acids - as hapten carriers, has gained favour in the past two decades.^{78f} The methodology is the same as for artificial haptens but there is the added advantage that the structure of the antigen is far better known. The immunogenicity of a synthetic polypeptide is less predictable than for natural proteins. Responses down to 451 dalton have been found, but some polypeptides on the other hand are not immunogenic at all. Immunogenicity is at least partly controlled by genetics, and the immune response will vary not only from species to species, but also within a species. Variation in the structure of a polypeptide increases its immunogenicity, with copolymers being better than a polymer of a single amino acid. Poly-L-lysine is not immunogenic, but if an accessible hapten is linked on, antibodies to both result.

The basic properties of an antigen then are:

- 1) It is immunogenic.
- 2) It possesses antigenic determinants.

Immunogenicity can be greatly enhanced by the use of adjuvants, which are non-specific immunity-stimulants, enhancing the immunogenic properties of an antigen or hapten-protein complex, or else acting on the cells involved in the immune response. A wide variety of compounds and

mixtures act in this manner, but the most effective general adjuvant⁸⁵ is "Freund's complete adjuvant", a water-in-oil emulsion containing killed Mycobacteria.^{75c,86} The means by which these work is not at all clear, and probably a number of mechanisms are triggered, but their effect on the immune system is dramatic, and synthetic polypeptides which are non-immunogenic will stimulate antibodies against themselves if used with an adjuvant.

Coupling Methods. The coupling method must provide a stable, covalent bond between the hapten and the protein. There are a number of reactive groups on a protein, but the reaction must be effective in the aqueous solvent. The hapten, when coupled, should also possess some detectable characteristic so that the degree of substitution can be determined; a chromophore absorbing at wavelengths longer than 280 nm would be suitable. Many methods could be devised, and the common ones have been summarized by Jonas.⁸⁷ Haptens may be coupled:

- 1) Via azo linkages (Scheme IV). The difficulties of incorporating an amino-substituted phenyl group on the hapten are compensated by a simple coupling reaction in which the bond, generally to tyrosine or lysine, also provides a suitable chromophore.

Scheme IV

- 2) Via ester and amide linkages (Scheme 1w). An acid can be coupled to the hydroxyl and amino groups of a protein using carbodiimides. The method can be extended by double ester linkages. Thus sterols with succinic anhydride give acid derivatives that can be coupled to proteins. A suitable chromophore is not provided by these ester links.

Scheme 1w

- 3) Via double amide linkages using toluene 2,4-diisocyanate (1.45)

Scheme 1x

Bonding is mainly to lysine

Other methods⁸⁷ include: thiolating the protein followed by acylation and alkylation, coupling with cyanuric chloride, and a number of methods suited to protein-protein coupling. The differing residues on a protein permit almost any hapten to be coupled via a suitable link that may also have the advantage of making the hapten more accessible.

Antibody-Antigen reactions

An antibody complexes with an antigen by forming a multitude of weak reversible bonds. The activation energy is very low and the rate of reaction is limited by diffusion. Affinities are generally high enough to effectively eliminate the free antigen from the blood. However, there is a considerable variation in the specificity of antibodies produced by an animal and some animals produce far more specific antibodies than others. The result is a distribution of affinities to a hapten, and, like most biological systems, variation limits precise description. Despite this, there are basic tests for antibody activity that are often used qualitatively, and can be made moderately quantitative.^{75d}

Precipitation. The antibody is always multivalent,,and an antigen is generally multivalent too. A mixture of antibody and antigen will form a cross linked lattice and if extensive, this will precipitate. As small antibody-antigen complexes are soluble, precipitation occurs at a maximum at equivalence (Scheme 1y). The antigen may have hundreds of haptens on it, but the effective valence, determined by steric considerations, will be quite low. If there is a large excess of antibody, there is little precipitate with the complexes being small, each surrounded by a shell of antibodies. If antigen is present in a

large excess, soluble complexes form once more, this time with an antigenic outer shell. Precipitation will occur under a variety of conditions and often testing is done with agar gel plates to give characteristic precipitin patterns.^{77b} If a hapten is to be tested, then as the serum contains antibody to both carrier and hapten, a different carrier is required to test for specific precipitation. If a precipitate is obtained so demonstrating hapten specificity, then cross-hapten reactions are very simple.^{84c} Since monovalent antigen would terminate a chain in the complex, a free hapten will do the same.

Scheme 1y

Thus if free hapten is mixed with an antibody-containing serum, and so blocks many antibody binding sites, when the carrier bound hapten is added, cross linking is severely restricted and little precipitate falls.

Indirect Haemagglutination Test.^{75e} As precipitation may be rather insensitive, a method of increasing the efficiency is to couple the test antigen to sheep erythrocyte, a procedure which increases the number of determinant groups that are linked together. The result is greatly enhanced agglutination and the method is one of the most sensitive methods for detecting antibodies against proteins or polysaccharides.

Complement Fixation Test.^{75f} This test uses the haemaglobin in red blood cells (erythrocytes) as an indicator for the presence of an antibody-antigen complex. The method uses a function of the immune system, namely the ability of an antibody, when bound to an antigenic cell, to make use of an agent, complement to rupture that cell. This principle, the "fixation" of complement by antigen-antibody complexes, can be used as a measure of either antigen or antibody. In the complement fixation test, two antigen-antibody complex systems are used. One, the test system, uses either a known amount of antibody in the presence of unknown antigen, or the reverse. If an antigen-antibody complex is formed, complement is "fixed" and is then unavailable for the second antigen-antibody system, the indicator system. The indicator system usually consists of sheep red blood cells (SRBC) and a rabbit antibody directed against SRBC. In the absence of an antibody-antigen complex in the test system, complement is not fixed, and is thus available for the indicator system. The complement reacts with the complex giving lysis of the SRBC, and release of haemaglobin into the test tube solution. SRBC which do not react form a red precipitate and in extreme cases, there is no red colouration in solution.

The "fixing" of complement is a complex process that has recently been largely elucidated. Complement is a set of nine proteins which interact in a cascade sequence.⁸⁸ The C_{H2} domain of the antibody (Scheme 1u) is altered in conformation by the antigen binding to the antibody, and it is to this domain that the first protein, C1, is attached, (Scheme 1z). The proteins are numbered C1 to C9, with lower case letters denoting fragments, e.g. C1q, and a bar indicating an enzyme complex, e.g. $\overline{C42}$, of C4 and C2. The enzyme C1 binds C4 to C2 to give $\overline{C42}$, which will then bind a major fragment of C3 to the target cell membrane. This is a new enzyme $\overline{C423}$, which activates the linking of C5, C6 and C7 to give $\overline{C567}$ which binds separately to the membrane. Finally C8 and C9 are linked in to give $\overline{C56789}$, which causes a lesion in the cell wall.

Scheme 1z

The lesion is about $100 \text{ \AA}$ in diameter, depending on the species, and the result is a net flow of ion and solvent into the cell, due to the Donnan effect, which swells and ruptures the cell, releasing haemoglobin in the case of sheep erythrocytes. Because the system is so effective, it is under strict control, with inhibitors limiting enzyme action, and the intermediate enzymes being rather unstable. $\overline{C42}$, for instance, has a

half life of 8 minutes at 37°C. The requirement of antibody specificity is necessary, for whereas it is desirable to bring invading cells to a timely end, attack on the animal's own blood cells would be distinctly unfortunate. The system also has a number of other desirable immunological features, but as these are not required for the test mechanism, they have not been discussed.

By cross-hapten studies, the degree of specificity of a mixture of antibodies can be estimated; in particular, the specificity to sporidesmin. Sporidesmin added to the test serum would bind to an antibody specific to it, and although this hapten-antibody complex would not cause loss of complement activity, addition then of antigen would be ineffectual as the antibody has already reacted. By this double negative system, cross hapten specificity can be examined. Having demonstrated antibodies specific to sporidesmin in guinea-pigs, the next steps would be to demonstrate high titre antibodies in sheep serum, and then to show *in vivo* protection against sporidesmin. These tests are not in the scope of the present work, but are the preliminaries to proving the effectiveness of any vaccine this work may produce.

Vaccines derived from naturally occurring sporidesmin. The toxin, sporidesmin-A, would seem the most likely precursor to a hapten for use in preparing a vaccine. Although there are difficulties with such a scheme, such as an inadequate supply of toxin, there have been studies made concurrently with this present work to prepare a hapten from the native toxin.⁸⁹ Ronaldson has coupled a sporidesmin-A derivative to a number of natural and synthetic polypeptides using transacylation of an ester.³⁰ Coupling was via the sulphur atoms, which has the advantage that almost all naturally occurring sporidesmins would be very similar to the hapten, (Scheme 12).

The hydroxyl groups are not alkylated since sulphur is a better nucleophile than oxygen. Transesterification preferentially occurs at the primary amines in the protein but both single and double amide links may occur. Although a monoester derivative from a dichloroacetate might have been preferable (to maintain the cage structure similar to the very toxic trisulphide, sporidesmin-E) the overall shape of the above hapten would be very similar to free sporidesmin. The synthetic polypeptide, poly-L-lysine, was used, for although not a good immunogen, it contained a large number of lysine residues, and was of a well-defined structure. For comparison, the very large protein, keyhole limpet haemocyanin, was also used because it is a very good immunogen.

Cross hapten studies showed that antibodies were produced that were specific to both free and bound sporidesmin, but the response was very weak, and the animals could not be regarded as effectively immunised. Two reasons were postulated, 1) a fault in the analytical method, which was still under development, or 2) sub-optimal sporidesmin-hapten concentration on the surface of the antigen. Other analytical procedures could test the first postulate, but the second postulate is more difficult to check. Increasing the number of haptens per antigen

would saturate all the lysine residues, some of which were needed for protein-protein coupling in the indirect haemagglutination test, used to analyse the results. Even if saturated, it is not certain if this is a sufficient amount.

It was considered that both a different testing procedure and a number of other haptens would help clarify the matter. This thesis is concerned with the preparation of those haptens, and the method of analysis utilised the complement fixation test.

Basic requirements in the synthetic work of this thesis

The immunological specifications of the compounds to be prepared are:

- 1) The compounds must bear a close resemblance to sporidesmin when coupled to a protein.
- 2) The compounds must possess a group suitable for covalent bonding to a protein.
- 3) The compounds should either absorb at wavelengths longer than 280 nm, or else have some other property to establish bonding to a protein has occurred.
- 4) A large number of allied compounds should be made for cross hapten studies.

Synthetic approach

The procedure adopted was to develop a suitably substituted benzenoid hapten and to test it. Intermediates from that preparation would be used to make an appropriate indole hapten.

Concurrent with the work on these two haptens would be work developing a pyrroloindole hapten which would closely resemble sporidesmin-A. The remainder of this thesis is therefore divided into three parts describing these three synthesis projects, and concludes with an analysis of the immunological results.

Part 2 Synthesis and Structural Elucidation of CompoundsRelated to the Hapten,2-Amino-5-chloro-3,4-dimethoxybenzylalcoholSection ASynthesis of the Hapten

The first object of the research was to prepare a monocyclic hapten suitable for coupling to a protein. As the benzenoid portion of sporidesmin-A (1.1) is common to all the sporidesmins (1.4-1.9), the synthesis of a suitably substituted benzene derivative would give a hapten related to the entire sporidesmin group. As a benzenoid hapten is much smaller than the optimum hapten size (that of a pentapeptide) the linking group and the amino acid residue to which the benzenoid hapten is bound, will also be expected to form part of the determining group on the antigen. The result is likely to be antibodies that have poor specificity for the sporidesmins. However, as the analytical methods for antibody testing were not well developed at that time, a simple hapten system, with a large number of related compounds for cross-hapten studies, was considered a useful target. To maintain the highest possible specificity for sporidesmin-A, the coupling group would have to extend from either the benzyl alcohol or amino group of the benzenoid moiety. Possible compounds, therefore, were the aminobenzyl alcohol (2.1), or a dimethylaniline derivative (2.2).

Sporidesmin-A

R is any suitable coupling group, see text.

The aminobenzyl alcohol (2.1) was selected because the arylamino group permits coupling to a protein, the azo coupling having the further advantage of forming a chromophore ($3\lambda_{\text{max}}$, 300-500 nm) which can be used to establish the presence of the hapten on the protein. Members of the class of compounds (2.2) have two limitations. Firstly the coupling group R must be limited to groups capable of reacting with a protein, but not with the hydroxyl group of another hapten, and R should also contain a suitable chromophore when linked. This excludes almost all the usual coupling methods except azo linking as in compound 2.1. Secondly, the members of the class (2.2) all possess a chiral center bearing the hydroxyl group, and any hapten would be produced as a racemic compound. If the enantiomers were not separated, then two sets of diastereoisomeric antigens would be produced, and two sets of antibodies stimulated. As all the sporidesmins (1.1, 1.4-1.9) have the same configuration at the corresponding chiral centre, the two sets of antibodies could possess very different affinities for the sporidesmins. The simplicity of hapten 2.1 therefore recommended it, and this became the first target molecule.

The most efficient route found for synthesising the hapten (2.1) is given in Scheme 2a. The starting material was cheap and readily available vanillin (2.3), a byproduct of paper pulp production.⁹⁰ The

Scheme 2a

chemistry of vanillin has been well characterised because of the problem of defining the structure of lignin. As the delignification of wood in pulp manufacture uses hypochlorite, the chloro derivatives are also well characterised, and the proton magnetic resonance (PMR) spectra,⁹¹ carbon-13 magnetic resonance (CMR) spectra,⁹² and ultraviolet and visible spectra⁹³ have all been reported. The chemistry is also well developed because many vanillin derivatives are physiologically active. The aryl dimethoxy or hydroxymethoxy functionality is found in compounds such as mescaline (2.12) and 3-methoxydopamine (2.13) which may act as adrenaline (2.14) mimetics. Vanillin itself is a flavouring agent and commercially available samples are very pure, although the melting point is rather broad (81-82.5°C) because under the conditions of manufacture it crystallises in two of four known forms.⁹⁴ A number of syntheses of vanillin are known, of which the best is probably that of Mottern,⁹⁵ and vanillin as starting material therefore implies a complete synthesis of any derived product.

Chlorination of vanillin in chloroform gave the 5-chloro product (2.4) in good yield. Chlorine gas was used, generated by the action of potassium permanganate on hydrochloric acid. Approximately one mole of chlorine (from 0.4 mole of permanganate) was piped so as to saturate the atmosphere above a very vigorously stirred solution of vanillin in chloroform. The chlorovanillin which precipitated (and which blocked

any immersed bubbler) was filtered off from the chilled solution.

Then the process was repeated on the filtrate. Although the procedure was tedious, final yields were good and recrystallisation from acetic acid gave a product melting in accord with the product of Raiford and Lichty.⁹⁶ 5-Chlorovanillin has also been prepared using t-butyl hypochlorite ($t\text{BuOCl}$) as the chlorinating agent, but the preparation of this reagent can be hazardous.^{97a}

Acetylation of 5-chlorovanillin according to the method of Raiford and Lichty⁹⁶ gave the triacetate (2.5) rather than the expected monoacetate (2.6), and this result has also been noted by Hodges and Taylor.²⁰ The monoacetate is generally obtained along with the triacetate, as a result of hydrolysis, and if the reaction is quenched in water rather than ice, the monoacetate (2.6) is the major product.

The triacetate (2.5) reacted with fuming nitric acid to give 4,O-acetyl-5-chloro-2-nitrovanillin (2.7), which is the same product as was obtained from the monoacetate.⁹⁶ As recrystallisation of the crude triacetate removed a considerable amount of the monoacetate, nitration should be carried out on the crude product to obtain the best overall yield of the nitrovanillin (2.7) from 5-chlorovanillin (2.4). As nitric acid has been used in both acetic acid and acetic anhydride solvents,⁹⁸ it may well be possible to nitrate the triacetate (2.5) in the acetylating medium, which would eliminate the isolation and lengthy drying period of the acetates (2.5 and 2.6). The 4-O-acetyl-5-chloro-2-nitrovanillin (2.7) was isolated both as the ethanolate reported by Raiford and Lichty,⁹⁶ and also as solvent-free crystals, which these authors had obtained by heating the ethanolate under vacuum. However if the ethanol is azeotropically removed by distillation with benzene, toluene or carbon tetrachloride, the solvent-free crystals are obtained on cooling.

Two equivalents of base are required for the hydrolysis of the nitroacetylvanillin (2.7) as both the derived phenate and acetate have to be neutralised. The principal product of the hydrolysis, 5-chloro-2-nitrovanillin (2.8) had the expected melting point. However, recrystallisation from the recommended⁹⁶ solvent, 20% ethanol, was found to be difficult and inefficient. Toluene and chloroform were found to be satisfactory solvents, but two different crystalline forms were obtained. Crystallisation of the crude vacuum dried product (2.8) from chloroform resulted in chunky prisms and subsequent chloroform recrystallisations gave this product. But if these crystals were dissolved in refluxing toluene from which the water-toluene azeotrope was removed, needles crystallised on cooling and this form was retained on subsequent chloroform recrystallisations. The critical difference that determined which form was obtained appeared to be trace quantities of water, since the original chunky prisms could be recovered on adding a slight amount of water to the chloroform. Both forms have the same melting point, but are easily characterised by their very different infrared spectra.

5-Chloro-2-nitrovanillin (2.8) was implicitly an objective of Hodges and Taylor²⁰ who intended to methylate it to obtain the compound 2.15 and then oxidise to give the acid they required. As their reaction sequence and their methods represent alternative pathways to the hapten precursor (2.11), an outline is given in scheme 2.2b.

Scheme 2b

In order to obtain 5-chloro-2-nitrovanillic acid (2.9) the vanillin (2.8) was oxidised by silver oxide to give the desired product in 100% yield. The method used was a variation on the method of Pearl⁹⁹ who prepared vanillic acid (2.20) using a molar equivalent of silver ion and vanillin in base. When the Pearl reaction was repeated, the yield of vanillic acid was only 52%, but on doubling the quantity of silver oxide, the yield increased to 70%. Similarly, 5-chlorovanillin (2.4) now gave 5-chlorovanillic acid (2.17) in 86% yield. The silver oxide methods are simpler and more efficient than the nitrile method of Raiford and Potter¹⁰⁰ which is that used in Scheme 2b.

Although doubling the quantity of silver oxide increased the yield, it is not certain what is the minimum theoretical quantity. The minimum would be two molar equivalents in a normal redox reaction (reaction A, scheme 2c), implying a 50% yield for equimolar amounts, or else if a Cannizzaro reaction took place, a 75% yield could be obtained from equimolar silver ion and aldehyde. (Scheme 2c).

Scheme 2c

Pearl has shown that the Cannizzaro reaction of vanillin will proceed if silver is present,¹⁰¹ and with formaldehyde, vanillyl alcohol or its dimers and polymers can be obtained.¹⁰² However, he also demonstrated that only half of the expected quantity of silver ion is needed, which he explains by a concurrent production of hydrogen as the reduced species, most of which is absorbed by the silver.⁹⁹

The quantity of silver oxide used to oxidise 5-chloro-2-nitrovanillin (2.8) may possibly be larger than the minimum needed for a quantitative yield. However, since an excellent method for regenerating silver oxide from silver by permanganate oxidation has been described by Pearl,¹⁰² the reaction is not wasteful. It is surprising that the method works at all, since stronger oxidising agents such as permanganate and chromic acid fail.¹⁰⁰

The methylation of 5-chloro-2-nitrovanillic acid (2.9) proceeds quantitatively (by t.l.c.) if carried out using an anhydrous method: The acid was added to a mixture of dimethyl sulphate, anhydrous potassium carbonate and acetonitrile heated under reflux for 10 minutes and the mixture was diluted with ammonia and water to give a 90% yield of the ester. The method is similar to those used for the methylation

of 5-iodovanillin¹⁰³ and for phenolic and acidic plant hormones,¹⁰⁴ for which the solvent used was acetone. Acetone was an unsatisfactory solvent for methylating the vanillins because of condensation of the acetone with the formyl group during the aqueous precipitation phase of the procedure. Tetrahydrofuran and dioxane prevented the reaction from going to completion, but another dipolar aprotic solvent, dimethyl formamide, was an excellent solvent requiring however mechanical stirring and heat control.

Of a number of methods of methylating vanillin,¹⁰⁵ the best has been considered that of Buck¹⁰⁶ which uses repeated cycles of dimethyl sulphate and alkali. Although quite suitable for the non-acidic phenols such as 5-chlorovanillin, it is unsatisfactory for the nitrovanillins, and in a number of runs using this method, 5-chloro-2-nitrovanillin (2.8) was methylated in yields below 30%. In contrast, the anhydrous method was found to be rapid and near quantitative with the nitrovanillins, and slow but still quantitative with 5-chlorovanillin. The reaction end point is easily detected both by disappearance of the orange phenate from the reaction mixture and by analytical thin layer chromatography (t.l.c.) which shows the disappearance of the slow moving vanillin, and the appearance of a single fast moving product. Thus although the method of Buck¹⁰⁶ may be competitive in terms of yield for the synthesis of 5-chloroveratraldehyde (2.21), the anhydrous method is simpler, and in the case of the nitrovanillins, gives a much higher yield.

The double methylation of the hydroxybenzoic acid (2.9) resulted in the efficient synthesis of the product of Hodges and Taylor,²⁰ methyl 5-chloro-2-nitroveratrate (2.10). Reduction to the amine was carried out largely as described by those authors to

give the amine (2.11) as the hydrochloride salt. This was easily converted to the free amine, which melted at 37-39°C in agreement with the literature value.

The scheme (2a) used in this work to obtain the ester (2.11) is a fusion of the work of Raiford and Lichty⁹⁶ and of Hodges and Taylor.²⁰ It is more efficient than the reaction sequence used previously (scheme 2b) because oxidation to the acid is achieved in a single quantitative step, and methylation of the 4-hydroxy and acid functions are also achieved in a single high yielding step. The improved general methylation procedure was particularly important since sizeable quantities of 5-chloro-2-nitroveratraldehyde (2.15) were required in the synthesis of indoles, described in part 3 of this thesis.

Reduction of methyl 2-amino-5-chloroveratrate (2.11) with lithium aluminium hydride in ether proceeded smoothly to give an oil which was homogeneous by t.l.c. and had the PMR and infrared spectra characteristic of the desired hapten, 2-amino-5-chloroveratryl alcohol (2.1), which had been previously obtained by an inefficient method (page 54). The oil, when first obtained, was characterised not only by physical data, but also by the formation of three crystalline derivatives, the dibenzoyl compound (2.22), the phenylboronate (2.23) and the 2-naphtholazo dye (2.24).

The first derivative (2.22), was prepared because the oil, which could be obtained clear, colourless and homogeneous by preparative t.l.c., did not crystallise after several months at 4°C, during which it slowly discoloured. The dibenzoyl compound (2.22) gave confirmatory analytical and spectral data, indicating that the desired product (2.1) had been obtained. Similar confirmatory data was found for the other two derivatives, although they were prepared for other purpose as well.

As the hapten (2.1) was to be linked to the antigenic protein by diazotisation and coupling with tyrosyl or lysine residues, there would be convenience in preparing a derivative of the hapten that was easily purified but which lost the protective grouping under the acid conditions of diazotisation. Triphenylboroxole (2.28), an acid labile protecting reagent which had generally given good crystalline derivatives of carbohydrates¹⁰⁷ was heated with the aminoalcohol in refluxing benzene with water removal to give the benzoxazaborinane (2.23). The compound was surprisingly stable, compared with its carbohydrate analogues, and could be recrystallised from isopropyl alcohol.

Compounds containing annular oxygen, boron and nitrogen are very rare. Recently Cragg and Weston¹⁰⁸ prepared the oxazaborinane (2.26) and also the dioxo analogue (2.29) has been prepared¹⁰⁹ (scheme 2d). The combination of these two syntheses would appear to be yet untried, and thus the compound (2.23) is the first example of a 3,1,2-benzoxazaborinane.

Scheme 2d ($\text{R}' = \text{NMe}_2, \text{NEt}_2, \text{SEt}$)

The benzoxazaborinane (2.23) could be easily hydrolysed in acidic methanol, and diazotisation carried out without isolation of the intermediate amine. This was established by preparing the azo dye (2.24) by dissolving the benzoazaborinane in warm acidic methanol, cooling to 4°C , and proceeding as with a standard diazotization.^{110a} Although the oil (2.1) was not subjected to elemental analysis, the satisfactory analyses returned by the three derivatives and the compatible spectral data on all four compounds leads to the conclusion that the compound (2.1) had been prepared.

Section B

Observations Resulting from the Hapten Synthesis

Reductions

The method by which the hapten (2.1) was first obtained was by reduction of the nitroaldehyde (2.15) with sodium borohydride then with iron in concentrated hydrochloric acid but this proved inefficient. Catalytic hydrogenation, to reduce both the nitro and aldehyde groups simultaneously, failed to give the required product. The reactions are outlined in scheme 2e.

Scheme 2e

5-Chloro-2-nitroveratraldehyde (2.15) was obtained in good yield by the general methylation procedure (page 86). The product has been made once previously, by Raiford and Floyd,¹¹¹ who gave the melting range as 51-52°C, for fine needles from dilute ethanol. The product obtained here melted at 83-84°C, if recrystallised from petroleum ether-carbon tetrachloride, and when that sample of chunky cubes was recrystallised from ca. 70% aqueous ethanol, the product was fine needles,

partially melting at 66°C, but crystallising again to the previous form and melting at 82-84°C. The fine needles had a broad "hydroxyl" band in the 3300 cm^{-1} region of the infrared spectrum, but as the PMR spectrum showed no trace of ethanol, the compound was not an ethanolate. As the series of reactions carried out by Raiford and Floyd establish the compound as definitely the 5-chloro-2-nitroveratraldehyde, the identity of the compound is not in doubt, but the melting point is open to dispute. The chloronitroveratraldehyde (2.15) (m.p. 83-84°C) obtained in the present work was structurally characterised both by CMR studies (page 79) and by the synthesis of a known indole, described in part 3.

As both the aldehyde and nitro groups of 2.15 had to be reduced, the aldehyde had to be reduced first to prevent the formation of Schiff bases by aldehyde-amine condensation. The benzyl alcohol (2.31), the product of the borohydride reduction of the aldehyde (2.15), is an oil, which was obtained quantitatively, as found by t.l.c. and PMR evidence. It was characterised by the preparation of its crystalline benzoate, which was obtained by the standard Schotten-Baumann method^{110b} of shaking the oil with aqueous alkali and benzoyl chloride. The good yield first obtained by this reaction was not found on repetition of the experiment, and use of the mild base, borax, was similarly unsuccessful, for although t.l.c. evidence showed moderate yield of the benzoate, and the crude reaction mixture was solid, it could not be recrystallised. Difficulty in obtaining an o-nitrobenzyl alcohol ester has been found before in a similar case¹¹² and has been ascribed to strong hydrogen bonding between the hydroxyl and nitro groups.^{97b} However, Williams et al found that in their case,¹¹² a good yield could be obtained by using sodium hydride in benzene as the base (scheme 2f). It is here suggested that the same method could be usefully employed in the benzylation of 2.31.

The conditions selected for the hydrogenation were hydrogen at room temperature and pressure over a 5% palladium on carbon catalyst, with the nitroaldehyde (2.15) in 0.5 M sulphuric acid in ethanol. Hydrogen molecule uptake close to the theoretical value of .4 equivalents was observed, but t.l.c. evidence showed that apart from starting material (2.15) and the nitrobenzyl alcohol (2.31), only products with mobilities less than the desired product (2.1) were present. When the reaction vessel size and the amount of solvent only were reduced by a factor of ten, thus increasing the concentration of starting material from 0.01 M to 0.1 M, the slow reaction became so slow, for some unknown reason, that the system was inoperable. When the large vessel size (100 ml) was retained, and the concentration increased to 0.05 M, reduction proceeded as normal, but when the reaction solution was concentrated and left to stand, a new crystalline material was obtained. This was shown, by mass spectral, analytical and spectral data, to be a trimeric condensation

product (2.34) of the aldehyde-amine (2.33). It is drawn as a *trans-trans* and planar system, but studies with Dreiding models showed that the *cis-trans* and *cis-cis* configurations were also possible.

An analogous parent compound (2.38) has been obtained from the condensation of *o*-aminobenzaldehyde (2.37) when a template transition metal has been used (Scheme 2g).¹¹³ It is interesting to note that the catalytic hydrogenation of *o*-aminobenzaldehyde is also difficult,¹¹⁴ and it is suggested that the *ortho*-amino group may hinder formyl reduction.

Scheme 2g

The trimer (2.34) was only isolated in 15% yield, and as a template was not present, the most likely product of the reaction is a polymer in which the imine bonds have been reduced to amines. This problem can be avoided if the formyl group is first reduced to the alcohol by borohydride. However, hydrogenation of the nitrobenzyl alcohol (2.31) failed to give the wanted hapten (2.1) and a number of products with low mobilities resulted from reduction. Apart from products resulting from only partial reduction of the nitro group, other byproducts could arise from dechlorination^{115a} or reduction of the benzyl alcohol to a toluene derivative.^{115b} It was because of these side reactions that rather weak catalytic conditions were used, but even so no formation of the desired hapten was ever observed. The

use of other catalysts such as Rhenium,¹¹⁶ was not attempted, and further hydrogenation experiments were abandoned.

General borohydride reduction procedures

In order to avoid Schiff base formation during the catalytic hydrogenation of the nitroaldehyde (2.15), an investigation of the borohydride reduction of vanillins and veratraldehydes was undertaken. Much of the preparative literature on the production of vanillyl and veratryl alcohols appeared before the development of sodium borohydride in 1953. For instance, a nitroveratryl alcohol was then obtained by the Meerwin-Pondorf reduction of a nitroaldehyde using aluminium *iso*-propoxide. Since then some work has been carried out using sodium borohydride. Brink¹¹⁷ reduced vanillin in 30 minutes to give vanillyl alcohol in high yield.* This short reaction time is recommended, as reaction for 24 hours gave a lowered yield and an increased amount of high melting byproduct. On the other hand Crooj¹¹⁸ reduced 5-chlorovanillin by a 30 hour reaction. As the vanillins and veratraldehydes differed greatly in their base solubility and reactivity to sodium borohydride two different procedures were developed.

The veratraldehydes were highly reactive and the reactions were complete within one minute when carried out in methanol below 0°C. The low temperatures are preferred for the nitroveratraldehydes as there was some discolouring of the product in room temperature reactions.

*Footnote: There is an error in the Chemical Abstracts summary of this paper, (64,9620e), and the results for vanillin $[3,4\text{-MeO(OH)C}_6\text{H}_3\text{CH}_2\text{OH}]$ and *iso*-vanillin $[4,3\text{-MeO(OH)C}_6\text{H}_3\text{CH}_2\text{OH}]$ should be exchanged.

For this same reason, minimum quantities of borohydride are to be preferred. Complete reduction was easily determined by analytical t.l.c. (silica gel, chloroform) and by the disappearance of the carbonyl absorption at ca. 1700 cm^{-1} . The reaction solutions were allowed to warm to room temperature to allow the methanol to react with excess borohydride, then the methanol was diluted with water to precipitate the product. None of the veratraldehydes that were reduced were readily soluble in cold methanol, but were dissolved by warming then rapidly cooling. If this caused precipitation of the starting material, the finely dispersed solid was not cooled further but treated with borohydride as usual.

The vanillins were quite slow to react. Vanillin itself was not fully reduced in 30 minutes as implied by Brink,¹¹⁷ whether in methanol or 10% sodium hydroxide solution. Since sodium borohydride reacts with methanol but is stable indefinitely in aqueous base,^{97c} all the vanillins were reduced in 10% sodium hydroxide solution. Vanillin and 5-chlorovanillin were reduced fully in 30 hours, but the nitrovanillins were reduced in 30 minutes, which demonstrates the ability of the electron-withdrawing nitro group to offset, to some extent, the effect of the phenate ion. The general scheme is given in scheme 2h, with six of the aldehydes that were reduced. Two of these products, the vanillyl alcohol (2.43) and the veratryl alcohol (2.31) have not been described previously.

The ease with which the nitrovanillyl alcohols, or the veratryl alcohols can be prepared means that borohydride reduction is a simple method for making a derivative of these compounds for further characterisation. It is probably the most simple and efficient means of making the derived alcohols, and dimer and polymer formation is not observed.

Aldehyde			Product	
R	X	Y		
H	H	H	Vanillin (2.3)	→ vanillyl alcohol (2.40)
H	H	Cl	2.4	→ 5-chlorovanillyl alcohol (2.41)
H	NO ₂	Cl	2.8	→ 5-chloro-2-nitrovanillyl alcohol (2.42)
CH ₃	H	H	Veratraldehyde (2.39)	→ veratryl alcohol (2.43)
CH ₃	H	Cl	2.21	→ 5-chloroveratryl alcohol (2.44)
CH ₃	NO ₂	Cl	2.15	→ 5-chloro-2-nitroveratryl alcohol (2.31)

Scheme 2h

Synthesis of unchlorinated analogues

For reasons connected with the preparation of indoles, as well as the study of the effect of chlorine substitution in cross-hapten reactions, a set of non-chlorinated analogues of some of the compounds already discussed was prepared. 2-Nitrovanillin (2.48) was synthesised ¹¹⁹ by the method of Julia et al. and methylated by the anhydrous potassium carbonate procedure (Scheme 2i).

Scheme 2i

When the crude nitroacetylvanillin (2.46) was hydrolysed, as well as the expected main product, 2-nitrovanillin (2.47), an alcohol insoluble fraction was obtained that proved to be the 6-isomer (2.49). This mixture and fractionation have been observed before.¹²⁰ 6-Nitrovanillin can otherwise be synthesised in good yield by nitrating the 4-benzyl ether of vanillin, followed by acid hydrolysis.¹¹⁹ 6-Nitrovanillyl alcohol (2.50) was prepared, to characterise further the isomeric byproduct (2.49).

The isomeric distribution observed in the nitration of acetylvanillin is nearly the reverse of what would be predicted from the behaviour of the analogues listed in scheme 2k. The free (4-hydroxy) vanillins chlorinate, brominate and nitrate in the 5-position,¹²¹ which is easily rationalised by postulating the formation of a stable ketone intermediate (Scheme 2j).

Scheme 2j

When the 4-hydroxyl group of vanillin is acetylated or methylated, chlorination or bromination now takes place in the 6-position. Such substitution is also true for acetylguaiacol (2.51), veratrole (2.52), acetyl creosol (2.53) and methylveratrole (2.54). Nitration in these last four cases also takes place in the 6-position, but, as has been shown, in vanillin derivatives it takes place at the 2-position, provided the 4-position is acetylated. Veratraldehydes nitrate, chlorinate and brominate in the 6-position, so that 2-nitration can be seen as an unusual reaction. From a comparison of the compounds in table 2.1, the requirements for 2-substitution are a 1-carbonyl-3-methoxyl-4-acetoxy arrangement as in structure 2.55, and a nitronium ion as electrophile.

The requirements for 2-nitration do not seem predictable by the standard theory of Wheland intermediates or by invoking any known neighbouring group effect. Since the symmetry of the 2- and 6-positions about the 1,4-axis is disturbed only by the methoxyl group, and since it appears that both benzoates and acetates in the 4-position are effective¹²¹ the crucial factor would seem to be an enhancement of a methoxyl effect by the carbonyl of the ester. This could occur by the acetate-carbonyl ring closing to form a 5-membered acetoxonium ion (Schemes 2k and 2l) but this mechanism would be possible at both

Compound	X	Y	Z	Charged electrophile		Main substitution position	Reference		Parent compound
2.51	H	Ac	H	Br	NO ₂	6	122	123	
2.52	H	Me	H	Br	NO ₂	6	124	122	
2.53	Me	Ac	H		NO ₂	6		125	
2.54	Me	Me	H	Br	NO ₂	6	126	127	
2.45	CHO	Ac	H	Br		6	121		
2.45	CHO	Ac	H		NO ₂	2		121	
2.39	CHO	Me	H	Br	NO ₂	6	128	129	
2.39	CHO	Me	Cl		NO ₂	6		*	
2.5	CH(OAc) ₂	Ac	Cl	Cl		6	96		
2.6	CHO	Ac	Cl		NO ₂	2		96	
2.18	COOMe	Ac	Cl		NO ₂	2		20	

Table 2.1

*this thesis

the 2-position (2.56) and the 6-position (2.57). Since 3-methoxybenzaldehyde (2.58) nitrates in the 2-position¹³⁰ an interaction between the formyl and methoxyl groups and the incoming nitronium ion must take place. A 4-methoxyl disturbs this whereas the acetate apparently does not. No sound reason is evident for the lack of reactivity of the 5-position, at least in the veratraldehyde cases, and a satisfactory explanation for the phenomenon has yet to be given.

Synthesis of 5-chloro-6-nitrovanillin and derivatives

During the hydrolysis of crude 4-O-acetyl-5-chloro-2-nitrovanillin (2.7), a small amount of a byproduct was obtained and provisionally identified as 5-chloro-6-nitrovanillin (2.61). Establishment of this structure proved difficult due to the paucity of reliable literature on related compounds, and was finally established by CMR studies described in the next section. The compound (2.61) was methylated to give 5-chloro-6-nitroveratraldehyde (2.62) which had previously been synthesised by Raiford and Floyd¹¹¹ from 5-chlorovertraldehyde (2.21). The reactions relating these compounds are given in Scheme 2m.

Scheme 2m

See experimental section for literature details.

The discrepancies in the melting points of the two chloronitroveratraldehydes (2.15 and 2.62) are particularly noteworthy since the structures of both were reliably established.¹¹¹ The 5-chloro-2-nitro isomer (2.15) has already been discussed on page 55.

To check on the literature synthesis of the 6-nitro isomer (2.62), as derived from 2.21, 5-chloroveratraldehyde (2.21) was nitrated under a variety of conditions. (Explicit experimental details were not given in the literature.^{111,131})

1) A product identical (by infrared spectroscopy) to the methylation product of 2.61 was obtained by adding 5-chloroveratraldehyde (2.21) to warm concentrated nitric acid. This confirms that nitration has occurred in the 6-position, since the 2-nitro isomer has been reliably characterised (page 55).

2) When 5-chloroveratraldehyde was added to fuming nitric acid at room temperature, which was about 15°C above the temperature implied in the literature preparation,¹¹¹ a different product was obtained which had no carbonyl stretching band in the infrared, and melted at 125-126°C. Elemental analysis implied that the product was a chlorodimethoxydinitrobenzene.

3) Under the same conditions as in 2), but with the temperature carefully kept at 0°C, which is the minimum temperature cited for the nitration,¹¹¹ a mixture of products was obtained. These were separated by preparative t.l.c. into three fractions, two of which were identified as being 5-chloro-6-nitroveratraldehyde (2.62) (63% yield) and the same chlorodimethoxydinitrobenzene (24%).

The third component, isolated in about 3% yield, was not identified, but from the infrared and mass spectral data, a mixture of compounds was deemed to be present; none of them contained a carbonyl group.

In a closely analogous reaction, Heacock and Hutzinger¹³² nitrated 5-iodoveratraldehyde (2.63) and found that the reaction did not give 5-iodo-6-nitroveratraldehyde (2.64) in the yield stated by Raiford and Wittmer.¹³¹ Under a variety of conditions, the veratraldehyde (2.64) was obtained in 44% yield at best, and three other non-aldehydic products were obtained as well.¹³² (Scheme 2n).

The products of the nitration of 5-chloroveratraldehyde (2.21) are likely to be the analogues of 2.64, 2.65 and 2.66. The two main products of the nitration are 5-chloro-6-nitroveratraldehyde (2.62) and a chlorodimethoxydinitrobenzene which most probably has the structure 2.69. The minor products may include the mononitrobenzene, 2.68. (Scheme 2o).

It is noteworthy that the single analysis figure given for their nitration product by Raiford and Floyd;¹¹¹ chlorine, 13.70%; is much closer to the calculated value for 2.69, 13.50%; than for 5-chloro-6-nitroveratraldehyde (2.62), 14.43%. It would appear that the low temperature fuming nitric acid reaction should be avoided, and 5-chloro-6-nitroveratraldehyde should be prepared using warm concentrated nitric acid.

The alcohols corresponding to 2.61 and 2.62, that is, 5-chloro-6-nitrovanillyl alcohol (2.70) and 5-chloro-6-nitroveratryl alcohol (2.71) were prepared, to characterise further the aldehydes 2.61 and 2.62. They were obtained by the standard reduction procedures (experimental section) and gave satisfactory analytical and spectral data.

Perchlorination of nitrovanillins

In an attempt to synthesise 5-chloro-6-nitrovanillin (2.61), 6-nitrovanillin (2.49) was chlorinated in hot methanol with a large excess of chlorine to give in good yield a product, Unknown-A, (2.72), that melted at 90-95°C. Unknown-A was a completely new substance that had little resemblance to its vanillin origins. Elemental analysis showed it to be a trichloro compound, even though only two positions on the benzene ring had been available for substitution (Scheme 2p). Heating was required to dissolve the starting material, and a large excess of chlorine was used because of difficulties in limiting chlorine production to precise molar amounts.

Scheme 2p

Repetition of the experiment using the more readily available 2-nitrovanillin (2.47) resulted in a compound, Unknown-B, (2.73), with similar spectral characteristics and elemental analyses to Unknown-A. Unknown-B could also be synthesised from 5-chloro-2-nitrovanillin (2.8), which therefore could be assumed to be a likely intermediate, as could 5,6-dichloro-2-nitrovanillin (2.74). An attempt to establish this, by passing a limited amount of chlorine into a solution of 5-chloro-2-nitrovanillin, resulted in the isolation of starting material (36%), Unknown-B (24%), a material of very low chromatographic mobility (ca. 23%) and a compound, which was not obtained pure, that ran somewhat

slower than Unknown-B on t.l.c. These results indicate that Unknown-B is very easily formed, and most of the reaction intermediates are short lived. The reactions are outlined in scheme 2q.

Scheme 2q

The PMR spectra of Unknown-A and -B are rather similar. Unknown-A has two medium field protons, 5.07 (1H,s) and 5.00 (1H,s), and a group of four methoxyls at shifts of 3.50, 3.42, 3.38 and 3.31 ppm. The spectrum of Unknown-B is similar, 5.31 (1H,s) and 5.04 (1H,s) and 3.46, 3.41, 3.41, and 3.39 ppm, each (3H,s,OMe). For each unknown, a set of reactions can be postulated, each requiring a dichlorobenzene intermediate, (2.74 and 2.79).

Scheme 2s

Similarly for Unknown-A

From these schemes, there are two suggested structures possible for each unknown, but a rather different structure such as 2.84 can also be proposed. As a result of studying the CMR spectrum of Unknown-A, Gottlieb¹³³ suggested 2.83 and 2.84 as two possible structures, and favoured 2.83.

All the structures suggested above should undergo acid catalysed alcohol exchange. However, refluxing Unknown-B in acidic ethanol for one hour gave starting material on cooling the solution, and distillation at 200°C did not degrade the compound. Refluxing in basic methanol gave a yellow tar and no isolated products. The structures of the unknowns are still a matter for conjecture, as further attempts at elucidation were not carried out.

Synthesis of 2-Amino-5-chlorovanillin

Because the initial results of the immunological testing showed that the hydrophobic character of the haptens in use might be preventing immunisation, a scheme was devised to synthesise the more hydrophilic hapten, 2-amino-5-chlorovanillyl alcohol (2.87).

5-Chloro-2-nitrovanillin (2.8) was reduced with ferrous sulphate in ammonia to give orange to brown needles melting at about 137°C as previously described.⁹⁶ The product was found to be insoluble in refluxing toluene, but some degradation occurred during this treatment since on cooling the toluene filtrate, a small amount of a new compound (m.p. ca. 137°C) crystallised. Elemental analysis of the toluene recrystallised material indicated that it had the expected structure, 2.86. It was found that the intermediate orange needles could be prepared from the derived 2-amino-5-chlorovanillin (2.86) by recrystallisation from aqueous ammonia. The compound was unstable, however, and elemental analyses indicated that it was a mixture of the aminovanillin (2.86) and an ammonium phenate salt of the vanillin, with one mole of water of crystallisation (2.85). The reaction, therefore, appears to have proceeded through a salt which is not mentioned in the original literature.⁹⁶ (Scheme 2t).

Scheme 2t

Raiford and Lichty⁹⁶ recrystallised their product from water, and it is suggested here that this process would cause loss of the ammonia leaving the aminovanillin (2.86) to crystallise out. A similar, but less efficient process occurred during the toluene reflux, but the conversion is very simply achieved by recrystallisation from dilute acid.

Attempts to reduce the aminovanillin (2.86) to 2-amino-5-chlorovanillyl alcohol (2.87) failed (Scheme 2u). When borohydride reduction was attempted, polymerisation occurred, presumably with concurrent reduction of the imine bond to give a polyamine. The stronger reagent, lithium aluminium hydride, caused similar degradation, and the only compound isolated was starting material. As catalytic hydrogenation had been found slow and ineffective in the corresponding veratraldehyde series, the attempts at reduction were abandoned.

Scheme 2u.

Section CNuclear Magnetic Resonance StudiesProton magnetic resonance spectra

The proton magnetic resonance (PMR) spectra of all the vanillin and veratraldehyde derivatives that were prepared are listed in tables 2.2 to 2.9. Experimental conditions are given on page 85.

The spectra can be seen to be comprised of peaks falling into well defined regions.

1) The proton of the formyl group was always found in the low field region, $\delta 10.35-9.57$ (generally 9.8 ± 0.1).

2) The aromatic protons range from shifts of $\delta 7.8$ to $\delta 6.7$, apart from benzoyl derivatives, which have the characteristic grouping of two multiplets; (ortho-protons, $\delta 8.0-7.85$, meta- and para-protons, $\delta 7.5-7.3$). The only complex coupling patterns are those of the three aromatic proton systems, as in vanillin, and the monosubstituted vanillin derivatives are characterised by the coupling constants. 2-Substituted derivatives give $J_{5,6} = 8-9$ Hz, 5-substituted derivatives give $J_{2,6} = 1.5-2$ Hz and 6-substituted derivatives have no observable coupling. This useful generalisation does not apply however if the two aromatic protons have the same chemical shift, as occurs in 5-chlorovanillyl alcohol, but separation and splitting should be observable if a europium shift reagent is used, as in the similar case of benzyl alcohol.¹³⁴ The use of substituent constants to calculate the position of a resonance fails badly, as would be expected of a heavily substituted benzene.¹³⁵ Thus the introduction of chlorine into vanillin has little effect on the resonances of the aromatic protons (2.4 vs 2.3) but when introduced into 2-nitrovanillin (2.47), a large shift occurs in the resonance of the remaining proton (spectrum of 2.8). This implies that even given a good

Table 2.2 Vanillins

Compound	Substituents	CHO	Ome	OH	Aromatic	Solvent
2.3	-	9.75	3.88	6.96	7.50-7.30 (2H,m), 7.00 (H,d,J _{2,5} =9 Hz)	CDCl ₃
2.4	5-Cl	*	3.99	6.5	7.50 (H,d,j=2 Hz), 7.35 (H,d)	CDCl ₃
2.8	5Cl, 2NO ₂	*	3.86	7.3	7.89 (H,s)	d ⁶ -DMSO
2.47	2-NO ₂	9.57	3.80	**	7.56 (H,d,J=8 Hz), 7.13 (H,d)	H ⁶ -DMSO
2.49	6-NO ₂	10.04	3.91	**	7.40 (H,d,J<0.5 Hz), 7.25 (H,d)	d ⁶ -DMSO
2.61	5-Cl, 6-NO ₂	9.71	3.97	**	7.62 (H,s)	d ⁶ -DMSO
2.85	2-NH ₂ , 5Cl	9.21	3.58	5.0(b) [†]	7.04 (H,s)	d ⁶ -DMSO
2.86	2-NH ₂ , 5Cl	9.56	3.67	4.6(b)	7.33 (H,s)	d ⁶ -DMSO

* corrected for paper expansion ** not observed † (ca. 8H, bs; NH₄, NH₂, H₂O)

Table 2.3 Acetyl vanillins

Compound	Substituents	CHO	Ome	4-OAc	Aromatic	Solvent
2.6	5-Cl	* 9.85	3.88	2.36	7.50 (H,d,J=2 Hz), 7.36 (H,d)	CDCl ₃
2.7	5-Cl, 2-NO ₂	* 9.84	3.92	2.41	7.75 (H,s)	CDCl ₃
2.45	-	* 9.85	3.84	2.29	7.50-7.35 (2H,m), 7.15 (H,d,J _{2,5} =8 Hz)	CDCl ₃
2.46	2-NO ₂	* 9.86	3.91	2.37	7.75 (H,d,J=8 Hz), 7.34 (H,d)	CDCl ₃
2.5	5-Cl	* 7.68**	3.87	2.36	7.32 (H,d,J=2 Hz), 7.09 (H,d)	CDCl ₃
	5-Cl (continued)			2.11	(6H,s,OAc)	CDCl ₃

* corrected

** CH(OAc)₂

Table 2.4 Veratraldehydes

Compound	Substituents	CHO	Ome	Ome	Aromatic	Solvent
2.15	5Cl, 2NO ₂	* 9.77	4.04	3.98	7.07(1H,s)	CDCl ₃
2.21	5Cl	* 9.82	3.94	3.91	7.46(H,d,J=1.5 Hz), 7.33(H,d)	CDCl ₃
2.39	-	9.67	3.88	3.85	7.29-7.25(2H,m), 6.86(1H,d,J=8 Hz)	CDCl ₃
2.48	2NO ₂	9.69	3.99	3.89	7.59(H,d,J=8.5 Hz), 7.12(H,d)	CDCl ₃
2.62	5Cl, 6NO ₂	* 9.79	4.01	4.01	7.37(1H,s)	CDCl ₃
2.88	6NO ₂	*10.35	4.01	4.01	7.57 and 7.36(2H,2d,J<0.5 Hz)	CDCl ₃

* corrected.

Table 2.5 Vanillyl alcohols

	ArCH ₂ O-	Ome	ArOH	Aromatic	BnOH	Solvent
2.40	-	* 4.61	3.91	**	6.95-6.85(3H,m)	3.91(s) CDCl ₃
2.41	5-Cl	4.34	3.75	8.9(b)	6.76(2H,s)	5.0(bs) d ⁶ -DMSO
2.42	5-Cl, 2-NO ₂	4.39	3.80	**	7.24(1H,s)	4.4(bs) d ⁶ -DMSO
2.50	6-NO ₂	4.75 [†]	3.86	9.65	7.42(H,d,J<0.5 Hz), 7.21(H,d)	5.3(bd) [†] d ⁶ -DMSO
2.70	5-Cl, 6-NO ₂	4.50	3.91	**	7.30(1H,s)	* CH ₃ CN

* corrected

[†] (doublet, J=5 Hz)

** not observed

Table 2.6 Veratryl alcohols

Compound	Substituents	ArCH ₂ O-	Ome	Ome	Aromatic	BnOH	Solvent
2.1	2-NH ₂ , 5-Cl	*	4.48	3.82	3.81	6.80 (1H, s)	CDCl ₃
2.31	5-Cl, 2-NO ₂		4.49	3.92	3.88	7.22 (1H, s)	CCl ₄
2.43	-	*	4.50	5.75	3.75	6.90-6.75 (3H, m)	CDCl ₃
2.44	5-Cl	*	4.51	3.80	3.80	6.88 (H, d, J=2H), 6.79 (H, d)	CDCl ₃
2.71	5-Cl, 6-NO ₂	*	4.62 [†]	3.95	3.89	7.05 (1H, s)	CDCl ₃

* corrected

[†] (doublet, J=6 Hz)

** not observed

Table 2.7 Vanillic acids

		COOH	Ome	ArOH	Aromatic	
2.9	5-Cl, 2-NO ₂	7.1	3.84	7.1	7.75 (1H, s)	d ₆ -DMSO
2.17	5-Cl	**	3.89	**	7.53 (H, d, J=2 Hz), 7.41 (H, d)	d ₆ -DMSO
2.20	-	9.7	3.84	**	7.55-7.36 (2H, m), 7.84 (1H, d, J=9Hz)	DMSO

** not observed

Table 2.8 Methyl veratrates**

		COOMe	Ome	Ome	Aromatic	ArNH ₂
2.10	5-Cl, 2-NO ₂	*	3.89	4.00	3.95	7.81 (1H, s)
2.11	2-NH ₂ , 5-Cl		3.85	3.77	3.77	7.47 (1H, s), 5.9 (2H, bs)

* corrected

** The methoxyl resonances need not be as assigned.

Table 2.9 Miscellaneous

Description		
2.22	Dibenzoyl of haptan 2.1	* 8.5(1H,bs,NH); 8.0-7.85, 7.50-7.35(10H,2m,benzoyl); 7.33(1H,s,ArH); 5.82(2H,s,ArCH ₂ O-); 3.87(6H,s,OMe) CDCl ₃
2.25	Phenyl boronate of haptan 2.1	* 7.8-7.6, 7.4-7.25(5H,2m,phenyl); 6.68(1H,s,ArH), 6.2(1H,bs,NH); 5.10(2H,s,ArCH ₂ O-); 3.95 and 3.85(6H,2s,OMe) CCl ₄
2.24	2-Naphthol azo dye of 2.1	* 8.16, 7.8-6.9(ca. 7 protons,ArH), 5.52(2H,s,ArCH ₂ O-), 4.07(3H,s,OMe), 3.95(3H,s,OMe) d ⁵ -pyr
2.32	benzoate of 2.31	8.0-7.8, 7.5-7.25(5H,2m,benzoate), 7.25(1H,s,ArH), 5.21(2H,s,ArH ₂ O-), 3.97(3H,s,OMe), 3.91(3H,s,OMe) CCl ₄
2.34	Trimer	* 7.04, 7.01 and 6.89, each (H,s,ArH); 5.46(2H,s,ArCH=N-), 4.02-3.93(ca.21H,m,6MeO,NH,CH ₂ (q)); 1.46(3H,t,CH ₃) CDCl ₃
2.69	chlorodinitroveratrole	* 7.68(1H,s,ArH), 4.04(3H,s,OMe), 3.98(3H,s,OMe) CDCl ₃
2.72	Unknown-A	* 5.07 and 5.00(2H,2s); 3.50,3.42,3.38 and 3.31(12H,4s,MeO) CDCl ₃
2.73	Unknown-B	* 5.31 and 5.04(2H,2s); 3.46,3.41,3.41 and 3.39(12H,4s,MeO) CDCl ₃

* corrected for paper expansion in humid conditions.

Abbreviations: d⁶-DMSO = hexadeuterodimethylsulphoxided⁵-pyr. = pentadeuteropyridine

s = singlet, d = doublet

t = triplet, q = quartet

b = broad, J = coupling constant (in Hertz)

All values given as shifts downfield relative to tetramethylsilane (TMS).

set of model tetra-substituted compounds, the identification of a penta-substituted benzene by calculating the shift of the remaining proton cannot be achieved simply.

3) The aromatic methoxyl protons resonated at shifts between $\delta 4.03$ to 3.77 . In the case of veratraldehyde derivatives, the peaks could not be assigned to particular methoxyl resonances.

4) The 4-O-acetyl protons of the acetylvanillins were found to resonate between $\delta 2.4$ and 2.3 , and were quite distinct from the benzyldene diacetate resonance at $\delta 2.11$ (compound 2.5).

5) The phenolic protons of the vanillins resonated with shifts of about $\delta 7$, or else were not observable. The acid protons were variable, but at low field, and the benzyl hydroxyls generally were found at a shift of about $\delta 4$ as broad singlets, although 5-chloro-6-nitroveratryl alcohol should have possessed a broad triplet. The hydroxyl protons were readily exchanged with deuterons on shaking with deuterium oxide, and as the benzyl hydroxyl protons often had the same shift as the methoxyl resonance, this technique in conjunction with integration confirmed their position.

Most of the peaks in the spectra could be assigned using the five general points above, and the remaining proton resonances, if any, were then easily assigned. An exceptional group were the methyl veratrates (table 2.8) where the methoxyl of the ester could not be distinguished from the aromatic methoxyls.

The spectra of vanillin (2.3), vanillyl alcohol (2.40), veratraldehyde (2.39) and veratryl alcohol (2.41) have been reported in the literature^{91,136,137a}, and no serious discrepancies were found on comparing the above results with the published data.

Carbon-13 nuclear magnetic resonance spectra

The CMR spectra of sixteen compounds in the above benzenoid series were recorded and the results are presented in tables 2.10-2.12.

Table 2.10 CMR shifts of substituted vanillins^a

Compound:	2.3	2.4	2.47	2.8	2.61	2.49
Carbon						
C(1)	128.7	128.0	118.3	118.3	118.4	123.2
C(2)	110.6	109.1	143.1	140.9	113.5	110.5
C(3)	148.0	148.6 ^b	139.3	141.9	149.0	151.6
C(4)	152.9	148.7 ^b	157.2	153.7	149.0	150.8
C(5)	115.3	120.0	117.9	123.3	112.8	110.9
C(6)	126.0	125.4	130.6	130.6	141.8	143.5
CHO	190.7	190.0	187.6	187.2	187.1	187.9
3-OMe	55.6	56.3	61.5	62.7	56.8	56.3

^a Recorded for solutions in hexadeuterodimethylsulphoxide, with shifts given downfield from TMS by: $\delta(\text{TMS}) = \delta(\text{d}^6\text{-DMSO}) + 39.5 \text{ ppm}$.

^b Assignment of signals may be interchanged.

Table 2.11 CMR shifts of substituted veratraldehydes^a

Compound:	2.39	2.21	2.48	2.15	2.62	2.88
Carbon						
C(1)	129.8	132.1	120.1	122.9	123.4	125.2
C(2)	108.7	109.4	144.2	142.6	110.2	109.5
C(3)	149.2	154.0	140.8	146.0	154.4	152.8
C(4)	154.1	150.4	158.2	155.0	150.4	152.0
C(5)	110.1	128.5	112.7	131.0	121.0	106.9
C(6)	126.4	125.4	128.2	126.6	144.2	143.5
CHO	190.4	189.6	185.6	184.4	184.7	187.2
3-OMe	55.9 ^b	56.0	62.8	62.6 ^c	56.6	56.6
4-OMe	55.7 ^b	60.7	56.6	61.3 ^c	61.3	56.6

^a Recorded for solutions in deuteriochloroform, with shifts given downfield from TMS by: $\delta(\text{TMS}) = \delta(\text{CDCl}_3) + 76.9 \text{ ppm}$.

^b Assignment of signals may be interchanged.

Table 2.12 CMR shifts of acetylvanillins^a

Compound:	2.45	2.6	2.46	2.7
Carbon				
C(1)	134.8	134.5	125.1	125.4
C(2)	110.6	109.2	144.7	143.3
C(3)	151.5	153.0	144.1	146.0 ^b
C(4)	144.5	141.4	148.8	145.9 ^b
C(5)	123.0	128.8	125.9	131.8
C(6)	124.1	124.3	126.4	126.1
CHO	190.5	189.5	185.7	184.4
3-OMe	55.7	56.3	62.8	63.0
<u>CO</u> me	167.8	166.9	167.3	166.2
CO <u>Me</u>	20.3	20.1	20.6	20.0

^a Recorded for solutions in deuteriochloroform, with shifts given downfield from TMS by: (TMS) = (CDCl₃) + 76.9 ppm.

^b Assignment of signals may be interchanged.

Vanillins

Veratraldehydes

Acetylvanillins

Vanillins	Veratraldehydes	Acetates	R ¹	R ²	R ³
2.3	2.39	2.45	H	H	H
2.4	2.21	2.6	H	Cl	H
2.47	2.48	2.46	NO ₂	H	H
2.8	2.15	2.7	NO ₂	Cl	H
2.61	2.62		H	Cl	NO ₂
2.49	2.88		H	H	NO ₂

The assignments were made by Professor E. Wenkert and associates, and are based on comparison with the literature and on a detailed analysis of carbon-hydrogen coupling constants over one and more bonds. The single frequency off-resonance decoupled spectra clearly identified carbons bearing a hydrogen atom, with two aromatic methines interacting to produce fine splitting of the carbon resonances. The other aromatic carbon resonances were assigned following specific decoupling of regions of the proton spectra: C(1) [coupled with the formyl proton, $J_{CH} = 25$ Hz], C(3) and C(4) (veratraldehydes) [coupled with the methoxyl protons], and carbons adjacent to aromatic methine groups [coupled with aromatic protons].

As a second means of establishing the structures of multi-substituted vanillin and veratraldehyde derivatives, the use of the additive substitution constants of Levy and Nelson¹³⁸ was investigated. The similar use of proton constants in the prediction of proton resonance was found impractical (see preceeding subsection), but the wide range of CMR aromatic resonances allowed the comparison of six carbon resonances, with a greater margin of error permitted than for a single proton resonance. The use of the additive substituent constants on the spectra of 2.3, 2.39 and 2.45 failed to predict accurately the resonance positions of derivatives, largely because aryl methoxyl groups are very sensitive to restriction to non-planar conformations. For example, the introduction of a 5-chloro substituent into vanillin moves the C(5) resonance downfield by 4.7 ppm, a value comparable to the literature value (6.2 ppm),¹³⁸ but the same substituent results in an 18.4 ppm shift in the veratraldehyde C(5) resonance.

A moderately successful prediction method was developed using the changes in shifts observed for the tetrasubstituted benzenes in comparison with their trisubstituted analogues. Thus for example to calculate the

spectrum of 5-chloro-2-nitroveratraldehyde (2.15), for each carbon resonance, the change in shift value between 5-chloroveratraldehyde (2.21) and veratraldehyde (2.39) was added to the shift value of the corresponding carbon of 2-nitroveratraldehyde (2.48). In general:

$$\delta C(n) [5\text{-Chloro-}x\text{-nitroV}] = \delta C(n) [5\text{-ChloroV}] + \delta C(n) [x\text{-nitroV}] - \delta C(n) [\text{unsubstituted V}]$$

V is vanillin, veratraldehyde or acetylvanillin; $n = 1, 2, \dots, 6$.

$x = 2$ or 6 ; $\delta C(n)$ = shift of carbon (n).

The spectrum found for a given compound was assigned as explained above, on the assumption that the structure given previously in this thesis was correct, and compared with the calculated spectrum (Table 2.12). On the assumption that nitration may have occurred in the 2- rather than the 6-position (or *vice versa*), the spectrum was reassigned by interchanging the C(2) and C(6) resonances, and compared with the spectrum calculated for this other isomer. The results, given in table 2.12, show that in the case of 5-chloro-2-nitro compounds, the spectrum could be calculated quite accurately and the given structures are confirmed. In the case of the 5-chloro-6-nitro derivatives, the difference between the alternatives is not so clear, although the results favour the assigned structures and so the method can be considered of very limited value in the case of compounds with two ortho substituents (Spectra 2.61 and 2.62).

The methoxyl carbon resonances fall into two ranges, 63-60 and 57-55 ppm, which provide another simpler and more general means of analysing structure. Inspection of tables 2.9 to 2.11 or comparison with alkyl substituted anisoles¹³⁹ shows that the 63-60 ppm group corresponds to ortho-ortho' disubstituted methoxyls, whereas the higher field group has an ortho hydrogen atom. 2-Substituted vanillins are

Table 2.12 Absolute discrepancies, $|\Delta|$, and mean discrepancy per carbon atom, $\Sigma|\Delta|/6$, between the calculated and observed chemical shifts for the ring carbons of the penta-substituted benzenes.

Spectrum	Calc. for structure	C(1)	C(2)	C(3)	C(4)	C(5)	C(6)	$\Sigma \Delta /6$
2.3	2.3	0.7	0.7	2.0	0.7	0.7	0.6	0.9
2.3*	2.61	4.2	21.6	10.3	7.1	7.7	2.0	8.8
2.61	2.61	4.1	4.5	3.2	2.4	2.8	1.1	3.0
2.61*	2.3	0.8	0.2	9.1	4.0	9.8	16.5	5.1
2.15	2.15	0.5	2.3	0.4	0.5	0.1	0.6	0.7
2.15*	2.62	4.6	16.4	11.6	6.7	5.7	0.1	7.5
2.62	2.62	4.1	0	3.2	2.1	4.3	1.7	2.6
2.62*	2.15	1.0	0.5	8.8	4.1	10.1	17.2	5.3
2.45	2.45	0.6	0	0.4	0.2	0.1	0.5	0.3

* the assignments for C(2) and C(6) have been reversed.

distinguished by the methoxyl resonance being in the 63-60 ppm range, and the same rule holds for acetylvanillin derivatives. For a veratraldehyde, a methoxyl resonance in the downfield range would imply either 2- or 5-substitution, but not both, which would be implied by both methoxyls resonances being in the downfield range. In turn this rule can be used as a method of assignment if the methoxyls fall in different ranges and the substitution pattern is known. The rule appears general and, in conjunction with methylation, is quite specific. As an example, the chloronitrovanillin (2.8) is substituted in the 2-position, and, as it was obtained from a chlorovanillin (2.4) which is not 2-substituted, the nitro group is in the 2-position. Methylation of 2.8 gives the veratraldehyde (2.15), which is 5- and 2-substituted, so the first compound (2.8) is 5-chloro-2-nitrovanillin. Confirmation of the structure of 5-chloro-6-nitrovanillin (2.61) can now be deduced simply from the spectra of the 5-chlorovanillin (2.4) and its nitro derivative (2.61), neither of which has a downfield methoxyl, or could be deduced independently of the previous example by noting that the methylation product (2.21) of 2.4 has a downfield methoxyl.

The three assignment techniques are all complementary, and the results of the analyses show that all sixteen compounds do have the structures attributed to them. The CMR resonances of the vanillyl and veratryl alcohols and acids corresponding to compounds in this series should be easily assigned using the techniques and results quoted here.

Section DExperimentalGeneral experimental methods and instrumentation

Microanalyses were Performed by Professor A. D. Campbell and associates, University of Otago. High resolution mass spectra were recorded by Dr G. J. Wright, University of Canterbury, on an AEI MS902 spectrometer, while low resolution mass spectra were recorded on a Micromass 12F instrument. Infrared spectra were recorded in deuteriochloroform solutions, unless otherwise stated, on an Hitachi-Perkin Elmer R20 60 MHz instrument operating at 34°C with tetramethylsilane (TMS) as internal standard. The paper used for recording PMR spectra expanded in humid conditions and this was corrected either by using chloroform as a second internal standard ($\delta 7.27$)^{137b} or else by offsetting TMS by 9 ppm downfield and recording the position. Correction values ranged from 0.4 to 2.3%, with an average of 1.6%. CMR spectra were recorded on a Varian XL-100-15 spectrometer operating at 25.2 MHz in the Fourier transform mode, by Professor E. Wenkert and associates, of Rice University, Texas. The abbreviations, in parentheses, s-singlet, d-doublet, t-triplet, q-quartet, m-multiplet, b-broad, J-coupling constant, follow the proton count and precede the proton assignment, if given. Merck Kieselgel GF₂₅₄ was used for t.l.c.; analytical plates were made to a thickness of 0.25 mm, and preparative plates to a thickness of 0.75 mm. Melting points are uncorrected and were determined using either a Reichert or Gallenkamp Hot Stage melting point apparatus.

Solvents were purified according to Perrin, Armarego and Perrin,¹⁴⁰ and the petroleum ether used throughout this work is the fraction distilling from 60-80°C. Methylations and reductions, if cited as being carried out by a general procedure, are the procedures below.

General methylation procedure for a vanillin

To dimethyl sulphate (12 ml, 127 mmole), in acetonitrile (50 ml) (distilled from calcium hydride) was added anhydrous potassium carbonate (25 g, 180 mmole). The vanillin derivative (100 mmole) was added and the mixture heated under reflux until analysis by t.l.c. (silica gel, CHCl_3) showed no starting material remained, and a single product had formed. (In the case of nitrovanillins, this is also shown by the disappearance of the red phenate, which takes about 10 minutes. For less acidic phenols, 2 hours reflux may be required). The mixture was allowed to cool, then poured into a stirred solution of 2 M ammonium hydroxide (100 ml, 200 mmole) which caused the separation of the product as an oil which generally rapidly solidified. The crude product was filtered off and dried under vacuum, then recrystallised from ethanol.

General borohydride reduction procedures

1) Veratraldehydes.— The veratraldehyde (10 mmole) in methanol (10 ml) was cooled to -10°C . Sodium borohydride (0.4 g, 10.8 mmole) was added slowly to the stirred mixture, the solution was allowed to warm to room temperature, then poured into ice water (20 ml). The precipitate that formed was filtered off and dried under vacuum. If the product was an oil, it was extracted from the diluted, salted solution with chloroform (10 ml, twice), dried with sodium sulphate and the filtered solution evaporated under vacuum to give the solvent-free oil.

The large excess of borohydride was not needed, and if the reagent was fresh, one third of the stated amount was used. Completion of the reaction is easily determined by analytical t.l.c. (silica gel, CHCl_3).

2) Vanillins.- The vanillins require longer reaction times (30 minutes for nitrovanillins to 30 hr for vanillin), and since methanol reacts with sodium borohydride, the solvent used was 2.5 M sodium hydroxide in water. Otherwise, conditions are as for the veratraldehydes. The reaction is terminated by acidification, carried out in a fume hood since diborane is released, and the precipitated product filtered off and vacuum dried.

A sample of the crude product in both cases should be analysed for carbonyl absorption ($1670\text{--}1720\text{ cm}^{-1}$) in the infrared spectrum. If there is any carbonyl present, the crude product should be redissolved and treated with more borohydride.

Synthesis of compounds

The PMR spectra of the following compounds are recorded in the previous section.

4-Hydroxy-3-methoxybenzaldehyde (2.3) (vanillin).- This was obtained commercially from Ajax Chemicals Ltd, Sydney, and used without further purification. It had m.p. $81\text{--}82.5^\circ\text{C}$ {lit.⁹⁰ $81\text{--}83^\circ\text{C}$ }, max. 3140 , 1668 , 1590 , 1519 , 1438 , 1300 , 1265 , 1168 , 1150 , 1028 , 860 and 728 cm^{-1} . {For KBr disk spectrum, see Brink¹¹⁷}.

3,4-Dimethoxybenzaldehyde (2.39) (veratraldehyde).- This compound was obtained commercially from British Drug Houses (BDH), and recrystallised from ethanol to give white needles, m.p. $44\text{--}44.5^\circ\text{C}$, {lit.¹²⁹ 43°C }, u_{max} . 1678 , 1668 , 1583 , 1509 , 1428 , 1287 , 1270 , 1240 , 1136 , 825 and 735 cm^{-1} .

3-Chloro-4-hydroxy-5-methoxybenzaldehyde (2.4).- Vanillin (2.3) (152 g, 1.00 mole) was dissolved in chloroform (500 ml) then chlorine (ca. 1.2 mole) was slowly bubbled through the vigorously

stirred solution. The precipitate that formed was filtered off and washed with chloroform, and the combined filtrate and washings were treated with a further mole of chlorine. A second crop was obtained, combined with the first, and recrystallised from acetic acid to give 5-chlorovanillin (2.4) (153 g; 82%), m.p. 163-165°C {lit.,⁹⁶ 163°C}, ν_{max} . 3350, 1679, 1598, 1511, 1434, 1300, 1150, 1050, 858 and 686 cm^{-1} .

4-Acetoxy-3-chloro-5-methoxybenzylidene diacetate (2.5).-

5-Chlorovanillin (2.4) (100 g, 0.537 mole) in acetic anhydride (200 ml, 2.12 mole) and acetic acid (150 ml) was heated under reflux for 2 hours, cooled, then the solution was poured onto crushed ice (1 Kg) and stirred until the product had solidified. Recrystallisation from ethanol gave the triacetate (2.5), (131 g; 74%), m.p. 114-115°C {lit.,⁹⁶ 115-116°C}, ν_{max} . 1760, 1592, 1235, 1197, 1047, 1008, 901 and 857 cm^{-1} . The crude product contained some monoacetylated product, and this 4-acetoxy-3-chloro-5-methoxybenzaldehyde (2.6) was the major product when the reaction was quenched with water. Some monoacetate (2.6) slowly crystallised on top of the main reaction precipitate (2.5 and 2.6) to give white needles, which had m.p. 63-65°C {lit.,⁹⁶ 67°C}, ν_{max} . 1768, 1696, 1590, 1427, 1282, 1173, 1130, 1052, 865 and 725 cm^{-1} .

4-Acetoxy-3-chloro-5-methoxy-6-nitrobenzaldehyde (2.7).- The triacetate (2.5) (40 g, 0.12 mole) was added slowly to fuming nitric acid (100 ml), stirred in an acetone/dry ice bath to keep the reaction temperature below 0°C. After addition was complete (15 minutes) the deep red solution was stirred for a further 10 minutes, then poured onto crushed ice (500 g). The crude product was washed well with water, dried, and recrystallised from benzene to give the nitroaldehyde (2.7) (26 g, 78%) which had m.p. 110-111°C {lit.,⁹⁶ 112°C}, ν_{max} . 1781, 1703, 1382, 1269, 1175, 1020, 887, 855 and 793 cm^{-1} . A sample crystallised from ethanol gave the monoethanolate, m.p. 94-95°C {lit.,⁹⁶ 95-96°C}.

3-Chloro-4-hydroxy-5-methoxy-6-nitrobenzaldehyde (2.8).- The acetate (2.7) (27.4 g, 0.1 mole) was suspended in a solution of sodium hydroxide (8.5 g, 0.21 mole) in water (100 ml) and ethanol (100 ml), and the mixture was stirred at 50°C for 15 minutes. Insoluble matter was filtered off and, with vigorous stirring, the chilled filtrate was acidified slowly with concentrated hydrochloric acid. After cooling for several hours at 4°C, the precipitated phenol (2.8) (21.6 g, 93%) was filtered off and dried under vacuum. Recrystallised from chloroform the chunky prisms had m.p. 137-138°C {lit.,⁹⁶ 137°C}, ν_{max} . 3330, 3200(b), 1695, 1687, 1594, 1555, 1266, 1149, 1088, 1004 and 857 cm^{-1} . A sample recrystallised from toluene gave needles, also m.p. 137-138°C, but had ν_{max} . 3190(b), 1680, 1542, 1304, 1217, 1142, 1057, 1000, 920, 810 and 766 cm^{-1} . Recrystallisation of the needles from chloroform did not change their form or IR spectrum, but recrystallisation from chloroform with a trace of water gave the chunky prisms once more.

3-Chloro-4-hydroxy-5-methoxy-6-nitrobenzoic acid (2.9).- Wet, freshly prepared, silver oxide [from silver nitrate (3.57 g, 21 mmole)⁹⁹] was suspended in water (25 ml), sodium hydroxide (2 g, 50 mmole) was added and the mixture heated to 50°C. The aldehyde (2.8) (2.31 g, 10 mmole) was added, the mixture stirred for 15 minutes, then the mixture was filtered and the silver residues washed with hot water. The combined washings and filtrate were treated with sulphur dioxide until the brown solution became yellow, 5 M hydrochloric acid (60 ml) was added, and the solution left at 4°C for 16 hours, after which the precipitated acid (2.9) (2.47 g, 100%) was filtered off, washed with cold dilute hydrochloric acid and dried under vacuum. Recrystallised from chloroform-ethanol, it had m.p. 192-194°C {lit.,²⁰ 197-199°C}, ν_{max} . 3580, 3510, 2620(b), 1689, 1549, 1413, 1240, 1030, 855 and 820 cm^{-1} .

Methyl chloro-4,5-dimethoxy-6-nitrobenzoate (2.10). - To dimethylsulphate (20 ml, 0.21 mole), acetonitrile (100 ml) and anhydrous potassium carbonate (35 g, 0.25 mole) was added the hydroxybenzoic acid (2.9) (20 g, 0.081 mole). The mixture was heated under reflux for 10 minutes, then allowed to cool. Ammonium hydroxide ($d = 0.880$) was added slowly until effervescence had almost ceased, then the solution was acidified with acetic acid (25 ml) and poured into chilled water whilst being vigorously stirred. After 15 minutes stirring, the precipitated ester (2.10) (20 g, 90%) was filtered off and dried under vacuum. Recrystallised from ethanol, it had m.p. $62-64^{\circ}\text{C}$, {lit., $^{20} 64^{\circ}\text{C}$ }; ν_{max} 1733, 1557, 1405, 1301, 1055, 977 and 891 cm^{-1} .

Methyl 2-amino-5-chloro-3,4-dimethoxybenzoate (2.11). - The nitroester (2.10) (20 g, 73 mmole) was dissolved in ethanol (500 ml), 2 M hydrochloric acid (250 ml) was added, and the mixture heated close to boiling. A stannous chloride solution, prepared by heating stannous chloride (20 g, 0.105 mole) and tin granules (20 g, 0.117 mole) in concentrated hydrochloric acid (200 ml) for two hours, was slowly added to the solution, which turned a deep yellow, then slowly began to lighten until after 100 minutes reflux it was a pale yellow. The solution was cooled by the vacuum distillation of solvent (ca. 300 ml) and the white precipitate which formed was filtered off and recrystallised from ethanol/dilute hydrochloric acid to give the hydrochloride salt of the amine (2.11) (16 g, 78%), m.p. 200°C (decomp.). This salt was neutralised by cold dilute sodium hydroxide solution, and the free amine extracted into chloroform (50 ml, twice), then the combined extracts were dried with sodium sulphate and evaporated to an oil. After some hours, crystals began to form, petroleum ether (20 ml) was added, and the amine (2.11) (10.9 g, 61%) was filtered off after 16 hours. A sample had

m.p. 37-39°C {lit.,²⁰ 37-39°C}, ν_{max} (as oil) 3470, 3350, 2910, 1690, 1606, 1585, 1310, 1125, 1026, 992, 891 and 797 cm^{-1} .

2-Amino-5-chloro-3,4-dimethoxybenzyl alcohol (2.1).— The aminoester (2.11) (5 g, 20 mmole) was dissolved in ether (35 ml) and added to a stirred mixture of lithium aluminium hydride (1.5 g, 40 mmole) in ether (25 ml) at a rate sufficient to maintain a gentle reflux. After the addition, which took 30 minutes, the stirring continued for 15 minutes, then water (2 ml), 15% sodium hydroxide (2 ml) and more water (6 ml) were successively added. The ether solution was filtered, the white residue washed with ether (20 ml), and the combined filtrate and washings evaporated under vacuum to an oil. The oil did not crystallise after several months at 4°C, but was homogeneous by t.l.c. (R_f 0.19, CHCl_3) and was characterised by spectral data and derivatives as the aminobenzyl alcohol (2.1) (4.3 g, 97%), ν_{max} (oil) 3360, 2910, 1615, 1472, 1247, 1090, 1027, 981 and 835 cm^{-1} .

2-Amino-di-N,O-benzoyl-5-chloro-3,4-dimethoxybenzyl alcohol (2.22).— The oil (2.1) (217 mg, 1 mmole), benzoyl chloride and aqueous sodium hydroxide were shaken together in a standard procedure^{110b} to give a solid which recrystallised from methanol to give the dibenzoate (2.22) (290 mg, 68%) which had m.p. 148-149°C, ν_{max} 3280, 1717, 1648, 1515, 1412, 1275, 1119, 1047, 976 and 706 cm^{-1} , (Found; C, 64.5; H, 4.9; N, 3.3; Cl, 8.3. $\text{C}_{23}\text{H}_{20}\text{ClNO}_5$ requires C, 64.9; H, 4.7; N, 3.3; Cl, 8.3).

3-Chloro-4,5-dimethoxy-6-nitrobenzaldehyde (2.15).— The nitrovanillin (2.8) (10 g, 43 mmole) was methylated by the general procedure to give the veratraldehyde (2.15) (10.1 g, 95%). Recrystallised from carbontetrachloride/petroleum ether, the chunky crystals had m.p. 83-84°C {lit.,¹¹¹ 51-52°C, (dilute ethanol)}, ν_{max} 1700, 1551, 1412,

1274, 1079, 1018, 963, 924, 863 and 670 cm^{-1} . Recrystallised from dilute ethanol, it formed fine needles, partially melting at 66°C and recrystallising to a more cubic form that melted $82\text{--}84^{\circ}\text{C}$, and had ν_{max} 3420(b), 1708, 1574, 1295, 1071, 1053, 1029, 965, 880 and 807 cm^{-1} .

3-Chloro-4-hydroxy-5-methoxybenzoic acid (2.17).— 5-Chlorovanillin (2.4) (5.6 g, 30 mmole) was oxidised as for vanillic acid (2.20) to give 5-chlorovanillic acid (2.17) (5.2 g, 86%). Recrystallised from water saturated with sulphur dioxide, the product had m.p. $250\text{--}252^{\circ}\text{C}$ {lit., ¹⁴¹ $252\text{--}254^{\circ}\text{C}$; ¹⁰⁰ $244\text{--}245^{\circ}\text{C}$ }, ν_{max} 3400, 2630, 1680, 1600, 1431, 1307, 1255, 1110, 1055, 883, 865 and 767 cm^{-1} .

4-Hydroxy-3-methoxybenzoic acid (2.20).— Vanillin (2.3) (1.52 g, 10 mmole) was oxidised by the silver oxide method of Pearl,⁹⁹ except that the mole ratios of aldehyde:silver ion:base were altered from (1:1:1.07) to (1:2.1:2.3). Full precipitation of silver oxide was checked by adding a little extra base to the supernatant liquid. The product was vanillic acid (1.18 g, 70%), m.p. $210\text{--}211^{\circ}\text{C}$ {lit., ⁹⁹ $210\text{--}211^{\circ}\text{C}$ }, ν_{max} 3460, 2610, 1678, 1601, 1441, 1300, 1237, 1201, 1029, 922 and 763 cm^{-1} .

In a previous trial in which the mole ratios were kept at the Pearl⁹⁹ values, vanillin (1.52 g, 10 mmole) reacted to give vanillic acid (0.87 g, 52%), and the filtrate was kept at 0°C and seeded, but no further acid crystallised.

3-Chloro-4,5-dimethoxybenzaldehyde (2.21).— 5-Chlorovanillin (2.4) (1.86 g, 10 mmole) was methylated by the general procedure, but with dimethylformamide stirred in a hot waterbath as the medium, to give 5-chloroveratraldehyde (2.21) (1.90 g, 95%). Recrystallised from petroleum ether, it had m.p. $55\text{--}57^{\circ}\text{C}$ {lit., ¹³¹ $54\text{--}55^{\circ}\text{C}$ }, ν_{max} 1695, 1577, 1500, 1422, 1286, 1142, 1050, 987, 856 and 675 cm^{-1} .

6-Chloro-1,2-dihydro-7,8-dimethoxy-2-phenyl-4H-3,1,2-*c*

benzoxazaborinane (2.23).— The aminobenzyl alcohol (2.1) (5 g, 23 mmole) was dissolved in benzene (50 ml), triphenylboroxole (2.28) (3 g, 9.6 mmole) was added, and the solution refluxed for 30 minutes with removal of water. The benzene was then removed by vacuum distillation to leave an oil which rapidly crystallised. Recrystallisation from 2-propanol gave the benzoxazaborinane (2.23) (5.65 g, 81%), m.p. 123–125°C, $\nu_{\max}$ 3430, 1613, 1490, 1396, 1239, 1115, 1044, 982, 777 and 689 cm^{-1} . (Found: C, 59.2; H, 5.1; Cl, 12.0; N, 4.7, $\text{C}_{15}\text{H}_{15}\text{BClNO}_3$ requires C, 59.4; H, 5.0; Cl, 11.7; N, 4.6%).

3-Chloro-6-(2-hydroxy-1-naphthylazo)-4,5-dimethoxybenzyl

alcohol (2.24).— The benzoxazaborinane (2.23) (152 mg, 0.50 mmole) was dissolved in methanol (8 ml) and concentrated hydrochloric acid (2 ml) by warming, then cooled in an ice-water bath. Sodium nitrite (55 mg, 0.8 mmole) in water (5 ml) was similarly chilled, mixed with the benzoxazaborinane solution, and left for 30 seconds. 2-Naphthol (78 mg, 0.54 mmole) in 10% sodium hydroxide (15 ml) which had been chilled also, was then added to the solution mixture, giving a red precipitate which was filtered off. Recrystallised from methanol, the azo dye (2.23) (130 mg, 70%) had m.p. 185–186°C, $\nu_{\max}$ 3400, 1620, 1490, 1280, 1027, 879, 836 and 750 cm^{-1} . (Found: C, 61.0; H, 4.7; Cl, 9.8; N, 7.6%; $\text{C}_{19}\text{H}_{17}\text{ClN}_2\text{O}_4$ requires C, 61.2; H, 4.6; Cl, 9.5; N, 7.5%).

Triphenyl boroxole (2.28).— Phenylboronic acid^{97d} (7.5 g, 56 mmole) was refluxed in benzene (50 ml) for 2 hours with concurrent removal of the water azeotrope, the volume of benzene was then reduced to 25 ml, cyclohexane (25 ml) was added, the solution brought to reflux, filtered hot and then cooled to 4°C. The voluminous white needles (2.28) had m.p. 213–214°C {lit.,¹⁴² 214–216, ¹⁴³ 190°C}, $\nu_{\max}$ 1604, 1446, 1354, 1312

1173, 1085, 1025 and 700 cm^{-1} , {lit.,¹⁴⁴ 1775, 1086 and 1023 cm^{-1} }.

3-Chloro-4,5-dimethoxy-6-nitrobenzyl alcohol (2.31).- The veratraldehyde (2.15) (491 mg, 2 mmole) was reduced by the general procedure to give an oil, which was mixed with water and extracted twice with ether (5 ml), dried with sodium sulphate and evaporated to dryness to give the alcohol (2.31) (474 mg, 96%) as an oil, which had ν_{max} (oil) 3400(b), 2950, 1562, 1538, 1480, 1411, 1374, 1295, 1031, 962 and 805 cm^{-1} .

3-Chloro-4,5-dimethoxy-6-nitrobenzyl benzoate (2.32).- The oil (2.31) was shaken with benzoyl chloride and 10% sodium hydroxide under standard conditions^{110b} to give the benzoate (2.32). Recrystallised from ethanol, the benzoate (2.32) had m.p. 86-87°C, ν_{max} 1727, 1569, 1536, 1409, 1280, 1265, 1251, 1115, 1065, 1016, 959 and 712 cm^{-1} . (Found: C, 54.6; H, 4.1; Cl, 10.1; N, 4.0%; $\text{C}_{16}\text{H}_{14}\text{ClNO}_6$ requires C, 54.6; H, 4.0; Cl, 10.1, N, 4.0%).

The repeated attempts at benzylation partially succeeded (t.l.c. showed up to ca. 40%) but the product could not be isolated by chloroform extraction, evaporation to a solid and recrystallisation, as no crystals formed in the ethanol even at -20°C with a seed crystal. When the method was repeated using the oil (2.31) (400 mg, 1.6 mmole), borax (2 g, 5.2 mmole) and benzoyl chloride (1 ml, 8.62 mmole) stirred for 24 hours, there was again t.l.c. evidence of extensive benzylation, and again no product crystallised out.

2,8,14-trichloro-6-ethoxy-5,6-dihydro-3,4,9,10,15,16-hexamethoxy-tribenzo[b,f,j][1,5,9]triazocyclododecine (2.34).- The nitroaldehyde (2.15) (1.27 g, 5 mmole) was dissolved in concentrated hydrochloric acid (2 ml) and ethanol (100 ml), then reduced by hydrogen (1 atm) over 5% palladium on charcoal (500 mg) for 20 hours, after which no further hydrogen was

adsorbed, and approximately 15 mmole had been consumed. The ethanolic solution was concentrated to 10 ml, left to stand overnight, and the crystals which formed were filtered off and dried under vacuum. The crystals were homogenous by t.l.c. [R_f 0.50 compared with starting material, R_f 0.77, silica gel, CHCl_3] and apart from this trimer (2.34) (150 mg, 15%), and starting material (2.15) the only other product present in the filtrate

was low-mobility material.

$C_8H_8ClNO_5$ requires C, 41.1; H, 3.5; Cl, 15.2; N, 6.1%.

3,4-Dimethoxybenzyl alcohol (2.43) (veratryl alcohol).-

Veratraldehyde (499 mg, 3 mmole) was reduced by the general procedure to give veratryl alcohol (472 mg, 94%) as a clear, colourless oil, $\nu_{\max}$ (oil) (3400(b), 2940, 1606, 1594, 1515, 1464, 1422, 1265, 1024, 1156, 1137, 1025, 810 and 764 cm^{-1} .

5-Chloro-3,4-dimethoxybenzyl alcohol (2.44).- 5-Chloroveratral-

dehyde (2.21) (201 mg, 1 mmole) was reduced by the general procedure to give 5-chloroveratryl alcohol (2.44) (192 mg, 95%) as a clear, colourless oil, $\nu_{\max}$ (oil) 3400(b), 2950, 1597, 1576, 1493, 1417, 1313, 1280, 1236, 1139, 1052, 1000 and 847 cm^{-1} .

4-Acetoxy-3-methoxybenzaldehyde (2.45).- Vanillin (2.3) (400 g,

2.63 mole) was acetylated by the method of Julia et al¹¹⁹ to give acetylvanillin (2.45) (495 g, 97%). Recrystallised from ethanol, it melted $76.5\text{--}77.5^\circ\text{C}$ {lit.,¹¹⁹ $76\text{--}77^\circ\text{C}$ }, $\nu_{\max}$ 1750, 1685, 1599, 1512, 1282, 1210, 1123, 1033, 911 and 746 cm^{-1} .

4-Acetoxy-3-methoxy-2-nitrobenzaldehyde (2.46).- Acetylvanillin

(2.45) (100 g, 0.52 mole) was added slowly to stirred fuming nitric acid (500 ml), kept below 0°C by a stirred ice/salt bath. After the addition was complete (30 minutes), the solution was stirred for a further 10 minutes, then poured over crushed ice. When the ice had melted, the precipitate was filtered off and dried under vacuum to give the nitroaldehyde (2.46) (122 g, 99%). Recrystallised from ethanol, it had m.p. $79\text{--}83^\circ\text{C}$ {lit.,¹⁴⁵ 85.5°C }, $\nu_{\max}$ 1780, 1703, 1553, 1277, 1180, 1163, 1013, 979, 872 and 753 cm^{-1} .

4-Hydroxy-3-methoxy-2-nitrobenzaldehyde (2.47).— 2-Nitroacetylvanillin (2.46) (150 g, 0.63 mole) was suspended in a mixture of methanol (70 ml) and water (100 ml), then sodium hydroxide (51 g, 1.27 mole) in water (100 ml) was added. The mixture was heated under reflux for 1 hour, then the resulting solution was cooled, filtered, and acidified with concentrated hydrochloric acid (150 ml, 2.5 mole), added slowly with vigorous stirring. The precipitate was filtered off and largely dissolved in ethanol, some insoluble crystals filtered off, and the filtrate dried with sodium sulphate. The ethanol was partly distilled off, with benzene being added until the clouding of the hot solution was slow to dissipate, then left to cool. The precipitated crystals were filtered off and recrystallised from chloroform to give 2-nitrovanillin (2.47) (90 g, 73%), m.p. 137–139°C {lit.,¹²⁰ 136°C}, $\nu_{\max}$ 3260, 1668, 1583, 1537, 1322, 1274, 1200, 1042, 979, 910, 833 and 785 cm^{-1} .

3,4-Dimethoxy-2-nitrobenzaldehyde (2.48).— The nitrovanillin (2.48) (16 g, 81 mmole) was methylated by the general procedure to give the nitroveratraldehyde (2.48) (17 g, 99%). Recrystallised from methanol, it had m.p. 63–64°C, {lit.,^{131,146} 55–56°C, ¹⁴⁷ 63.5–64°C}, $\nu_{\max}$ 1683, 1595, 1536, 1284, 1071, 1044, 961, 904 and 827 cm^{-1} .

4-Hydroxy-3-methoxy-6-nitrobenzaldehyde (2.49).— During the hydrolysis of acetylnitrovanillin (2.46) to give 2-nitrovanillin (2.47), an alcohol insoluble compound was formed after acidification, which was filtered off from the majority of the product, and recrystallised from chloroform-methanol to give 6-nitrovanillin (2.49) (2 g, 1.6%), m.p. 209–212°C {lit.,¹²⁰ 212°C}, $\nu_{\max}$ 3100(b), 1671, 1586, 1530, 1333, 1302, 1233, 1056, 919, 812 and 738 cm^{-1} .

4-Hydroxy-3-methoxy-6-nitrobenzyl alcohol (2.50).- 6-Nitrovanillin

(2.49) (197 mg, 1 mmole) was reduced by the standard vanillin procedure (1 hour) to give 6-nitrovanillyl alcohol (2.50). Recrystallised from toluene-methanol, the yellow crystals (175 mg, 83%) had m.p. 188-189°C, ν_{max} 3480, 3210(b), 1586, 1528, 1496, 1320, 1293, 1215, 1066 and 1037 cm^{-1} . (Found: C, 48.0; H, 4.5; N, 7.0; $\text{C}_8\text{H}_9\text{NO}_5$ requires C, 48.2; H, 4.6; N, 7.0%).

3-Chloro-4-hydroxy-5-methoxy-2-nitrobenzaldehyde (2.61).- The

crude product from a series of nitrations of acetylchlorovanillin (2.6) (300 g, 1.32 mole) was recrystallised from benzene to give the expected product (2.7). Concentration of the mother liquor gave an oil which was dissolved in alcohol, and an aqueous solution of 20% sodium hydroxide (200 ml, 0.4 mole) was added, and when the dark mixture had cooled, a precipitated orange solid (2.59) was removed. The orange solid was dissolved in dimethyl sulphoxide, acidified with concentrated hydrochloric acid, and diluted with alcohol to give colourless 5-chloro-6-nitrovanillin (2.61) (4.8 g, 1.6%). Recrystallised from alcohol, it had m.p. 192-193°C, ν_{max} 3120(b), 1688, 1554, 1515, 1316, 1223, 1179, 1102, 1018, 934, 858 and 822 cm^{-1} . (Found: C, 41.4; H, 2.6; Cl, 15.4; N, 6.1; $\text{C}_8\text{H}_6\text{ClNO}_5$ requires C, 41.5; H, 2.7; Cl, 15.3; N, 6.1%).

The orange solid (2.59) proved to be the sodiosalt, and could be obtained from a solution of the phenol (2.61) by adding one equivalent of sodium hydroxide in a small amount of water, and diluting with alcohol. The sodium vanillin-phenate (2.59) had no sharp melting point gradually decomposed on heating, but had ν_{max} 3590, 3250(b), 1667, 1580, 1540, 1511, 1234, 1095, 1087 and 866 cm^{-1} .

3-Chloro-4,5-dimethoxy-2-nitrobenzaldehyde (2.62).- The phenol

(2.61) (289 mg, 1.25 mmole) was methylated by the general procedure to give the veratraldehyde (2.62) (2.61 mg, 81%). Recrystallised from

carbontetrachloride several times, with light excluded, a sample had m.p. 134-135°C, {lit., ^{111,131} 122-123°C}, $\nu_{\max}$ 1705, 1568, 1541, 1359, 1319, 1301, 1205, 1090, 1016, 973, 900, 869 and 763 cm^{-1} . (Found, C, 44.3; H, 3.3; Cl, 14.5; N, 5.7; $\text{C}_9\text{H}_8\text{ClNO}_5$ requires C, 44.0; H, 3.3; Cl, 14.4; N, 5.7%).

Nitration of 5-chloroveratraldehyde (2.21) (analysis by infrared spectrometry).

a) The addition of 5-chloroveratraldehyde (2.21) in acetic acid to concentrated nitric acid ($d = 1.4$) at 0°C for 5 minutes resulted in the isolation of starting material (87%) only.

b) 5-Chloroveratraldehyde added to nitric acid ($d = 1.4$) at 30°C for 15 minutes gave mainly 5-chloro-6-nitroveratraldehyde (2.62) but also some starting material (2.21).

c) 5-Chloroveratraldehyde (2.01 g, 10 mmole) was added to nitric acid ($d = 1.4$ ml) and the mixture heated until effervescence began, when it was immediately removed from the heat, left for 15 minutes, then quenched in ice water to give 5-chloro-6-nitroveratraldehyde (2.62) (2.16 g, 88%) as the sole product.

d) 5-Chloroveratraldehyde (241 mg, 1.2 mmole) was added to fuming nitric acid (2 ml) at 0°C, left for 5 minutes, then poured into ice water (40 ml) stirred and left for 5 minutes, and then the white precipitate (286 mg) was filtered off and dried under vacuum. The precipitate was separated by preparative t.l.c. (silica gel, chloroform-petroleum ether 1:1) and three bands were collected, with the yellow baseline discarded. The low mobility material (R_f 0.2) was found to be 5-chloro-6-nitroveratraldehyde (2.57) (185 mg, 63%), which was recrystallised from toluene/petroleum ether to give almost white needles, m.p. 135-136°C. The middle band (R_f 0.3) was found to be the chlorodimethoxydinitrobenzene

(2.69) (76 mg, 24%) and the top band (R_f 0.5) (9.6 mg) was not identified. The infrared spectrum of the top band contained no carbonyl absorption and two mass spectra performed 1) immediately on heating the sample to 200°C and 3) after 10 minutes heating, differed greatly in relative intensities: m/e 262 (25%, base); 253 (5, 12%); 251 (14, 50%); 217 (base, 34%); 201 (12, 2%); 173 (4, 14%); 171 (11, 17%); only ^{35}Cl isotope given for all masses.

e) 3-Chloro-1,2-dimethoxy-4,5-dinitrobenzene (2.68).-

5-Chloroveratraldehyde (2.21) (401 mg, 2 mmole) was added to fuming nitric acid (10 ml) at room temperature (ca. 20°C), left for 5 minutes, then poured onto crushed ice and water (40 ml). The precipitate was filtered off, washed with water, and dried under vacuum. Recrystallised from methanol, 3-chloro-4,5-dinitroveratrole (2.68) (360 mg, 69%) was obtained as white needles, which had m.p. 125-126°C, ν_{max} 3095, 1550, 1348, 1303, 1064, 1020, 960, 889, 879, 754 and 734 cm^{-1} . (Found: C, 36.7; H, 2.8; Cl, 13.7; N, 10.7; $\text{C}_8\text{H}_7\text{ClN}_2\text{O}_6$ requires C, 36.6; H, 2.7; Cl, 13.5; N, 10.7%).

3-Chloro-4-hydroxy-5-methoxy-2-nitrobenzyl alcohol (2.70).- The 6-nitrovanillin (2.61) (694 mg, 3 mmole) was reduced by the standard procedure (1 hour) to give the nitrovanillyl alcohol (2.70). Recrystallised from dilute acidified alcohol, the white needles (595 mg, 85%) had m.p. 182-183°C, ν_{max} 3580, 3320(b), 1591, 1529, 1508, 1300, 1188, 1088, 999, 867 and 813 cm^{-1} . (Found: C, 41.0; H, 3.5; Cl, 15.4; N, 6.1; $\text{C}_8\text{H}_8\text{ClNO}_5$ requires C, 41.1; H, 3.5; Cl, 15.2; N, 6.0%).

3-Chloro-4,5-dimethoxy-2-nitrobenzyl alcohol (2.71).- The veratraldehyde (2.62) (737 mg, 3 mmole) was dissolved in warm methanol, rapidly cooled to room temperature and reduced by the standard procedure to give a dried precipitate. Recrystallised from carbontetrachloride in the presence of a little charcoal, and with light excluded, the

5-chloro-6-nitroveratryl alcohol (2.71) (590 mg, 79%) had m.p. 114.5–115.5°C, ν_{max} 3260(b), 1585, 1560, 1523, 1324, 1284, 1209, 1093, 1024, 892 and 859 cm^{-1} . (Found: C, 43.5; H, 4.0; Cl, 14.3; N, 5.7; $\text{C}_9\text{H}_{10}\text{ClNO}_5$ requires C, 43.7; H, 4.1; Cl, 14.3; N, 5.7%).

Unknown-A (2.72).— 6-Nitrovanillin (2.49) (594 mg, 2 mmole) was dissolved in methanol (50 ml) under reflux, and a large excess of chlorine (ca. 80 mmole) was bubbled in over 15 minutes. The resulting solution was evaporated to an oil, redissolved in methanol (5 ml) and acidified with concentrated hydrochloric acid to give a crude precipitate (820 mg) which was dried under vacuum. Recrystallised from methanol, the unknown-A (2.72) (694 mg, 92%) had m.p. 90–95°C; ν_{max} (nujol) 1552, 1212, 1131, 1086, 1063, 1032, 985, 785 and 744; (hexachlorobutadiene) 2955, 1769, 1468, 1363 and 1257 cm^{-1} ; R_f (CHCl_3) 0.8; PMR 5.07 (1H, s); 5.00 (1H, s); 3.50, 3.42, 3.38 and 3.31, each (3H, s); CMR 184.1 (C=O), 146.9 (C–Cl), 132.1 (C), 99.3 (CH), 99.2 (C), 72.7 (C–Cl), 55.2 (CH_3), 54.3 (CH_3), 52.5 (CH_3), 49.5 (CH) and 49.2 (CH_3), m/e 345.9654 and 347.9623, ($\text{C}_{10}\text{H}_{11}\text{Cl}_3\text{NO}_6$ requires 345.9652 and 347.9622) (Found: C, 35.0; H, 3.7; Cl, 27.5; N, 3.7; $\text{C}_{11}\text{H}_{14}\text{Cl}_3\text{NO}_7$ requires C, 34.9; H, 3.7; Cl, 28.1; N, 3.7%).

Unknown-B (2.73).— 2-Nitrovanillin (2.47) (87 mg, 5 mmole) was dissolved in warm methanol (30 ml), then chlorine (ca. 160 mmole) was bubbled into the cooling solution over an hour. The white solid which had separated, redissolved on the distillation of a little methanol and the residual chlorine, and white crystals formed on cooling the solution in stages to –20°C. The solid was filtered off and dried under vacuum to give Unknown-B (2.73) (1.297 g, 69%). The filtrate was diluted with water and ether extracted to give a yellow oil (466 mg) that on analysis by t.l.c., comprised at least five compounds, including unknown-B. A sample of Unknown-B recrystallised from carbontetrachloride-petroleum ether

had m.p. 131-132°C, $\nu_{\max}$ 1757, 1543, 1218, 1138, 1097, 1062, 997, 825 and 763 cm^{-1} ; PMR, 5.31 and 5.04 (2H, 2s), 3.46, 3.41, 3.41 and 3.39 (12H, 4s, 4MeO). (Found: C, 35.2; H, 3.6; Cl, 28.2; N, 3.8; $\text{C}_{11}\text{H}_{14}\text{Cl}_3\text{N}_2\text{O}_7$ requires C, 34.9; H, 3.7; Cl, 28.1; N, 3.7%)

Preparation of Unknown-B (2.73) with limited chlorine.-

5-Chloro-2-nitrovanillin (2.8) (3.48 g, 15 mmole) was dissolved under reflux in methanol (30 ml), then chlorine (30-35 mmole) was bubbled in. Unknown-B (0.98 g, 17%) was isolated by chilling the methanol, and starting material (0.86 g, 25%) was isolated by ether extraction, petroleum ether precipitation and toluene recrystallisation. An aliquot of the combined filtrates was separated by preparative t.l.c. (silica gel, 5% ethanol in chloroform) into three bands, which were weighed and analysed by t.l.c. This indicated that the products were:

- 1) Starting material, (36%)
- 2) Unknown-B, (24%)
- 3) Non-mobile material, (ca. 23%)
- 4) An unknown material that ran slower than Unknown-B, (ca. 17%), but which was not separated by the preparative chromatography. [Mole percent values are only approximate for 3) and 4)].

Stability of Unknown-B (2.73).- Unknown-B was recovered after refluxing in 20% concentrated hydrochloric acid in methanol for 1 hour, and cooling to crystallise and filter off the precipitate, and was unaltered (by IR analysis) after distillation at 200°C and 20 mm pressure. Reflux of unknown-B (200 mg) in ethanol (10 ml) and toluene sulphonic acid (20 mg) for 2 hours gave 87% recovery of crystallised Unknown-B. Reflux in 1 M hydrochloric acid in ethanol was similarly ineffective.

Unknown-B (379 mg, 1 mmole) and sodium hydroxide (80 mg, 2 mmole) in methanol (10 ml) was heated under reflux for 30 minutes, then acidified with hydrochloric acid and cooled. The methanol was distilled off under vacuum, the resulting oil dissolved in chloroform from which the sodium chloride was filtered, then the chloroform was distilled off to leave a clear orange oil. Analysis by t.l.c. showed no starting material remained (R_f 0.8), a considerable amount of very low mobility material present, and a compound that was mobile (R_f 0.2) but was not isolated (Silica gel, CHCl_3)

2-Amino-5-chloro-4-hydroxy-3-methoxybenzaldehyde (2.86).-

5-Chloro-2-nitrovanillin (2.8) (10 g, 43.2 mmole) was reduced according to Raiford and Lichty⁹⁶ to give orange needles which were filtered off, and the filtrate concentrated by vacuum distillation to give a second crop which was combined with the first to give the hydrate of ammonium 2-amino-5-chlorovanillin-phenate (2.85) (10.1 g, 98.6%). The salt (2.85) was dissolved in water under reflux, acidified and cooled to crystallise 2-amino-5-chlorovanillin (2.86) (7.65 g, 88%). Recrystallised from toluene, it had m.p. 138-139 °C {lit.,⁹⁶ 136-137°C}, ν_{max} 3460, 3340, 1639, 1616, 1543, 1537, 1774, 1176, 1091, 902, 874, 839, 748 and 697 cm^{-1} . (Found: C, 47.6; H, 4.1; Cl, 17.7; N, 7.3; $\text{C}_8\text{H}_8\text{ClNO}_3$ requires C, 47.7; H, 4.0; Cl, 17.6; N, 7.0%).

The salt could be regenerated if the free vanillin (2.86) (121 mg, 0.6 mmole) was suspended in water (3 ml) and concentrated ammonia (5 ml), the mixture heated under reflux until no solid was visible (about 5 minutes), then cooled to 4°C. The crystals (79.1 mg, 56%) which separated were filtered off and dried under vacuum. The filtrate was evaporated under vacuum to give a solid (60.6 mg, 43%) which was identical (by infrared spectroscopy) with both the first crop and with the salt (2.85) isolated by reduction of the nitrovanillin (2.8). The salt (2.85) melted

at the same temperature as the free aminovanillin (2.86) (138-139°C) but had $\nu_{\max}$ 3450, 3340, 3170, 2710, 1620, 1594, 1430, 1275, 1113, 1014, 865, 845, 776 and 714 cm^{-1} . [Two sets of elemental analyses were performed which showed decomposition of the salt (2.85) ($\text{C}_8\text{H}_{13}\text{ClN}_2\text{O}_4$) to form the free vanillin (2.86) ($\text{C}_8\text{H}_8\text{ClNO}_3$). Carbon, hydrogen and nitrogen were analysed simultaneously].

(Found: C, 41.8; H, 5.2; N, 11.2; $[0.82\text{C}_8\text{H}_{13}\text{ClN}_2\text{O}_4, 0.18\text{C}_8\text{H}_8\text{ClNO}_3]$ requires C, 41.7; H, 5.3; N, 11.1%).

(Found: C, 42.3; H, 5.1; N, 10.4; $[0.71\text{C}_8\text{H}_{13}\text{ClN}_2\text{O}_4, 0.29\text{C}_8\text{H}_8\text{NO}_3]$ requires C, 42.4; H, 5.1; N, 10.6%).

Attempted reduction of 2-amino-5-chlorovanillin (2.86).-

a) Lithium aluminium hydride (149 mg, 3.94 mmole) and the aminovanillin (2.86) (408 mg, 2 mmole) were mixed, tetrahydrofuran (5 ml) added, and the mixture gently refluxed for an hour, during which the solution slowly turned brown. Concentrated hydrochloric acid (3 drops) was added, the mixture filtered and the filtrate separated by preparative t.l.c. to give some dark non-mobile material which was not isolated and a broad band (R_f 0.2-0.34, CHCl_3) which was found by PMR spectra comparison to be the starting material (270 mg, 67%). There was some small extraneous peaks in the PMR due to impurities, but none occurred with a shift of about $\delta 5$.

b) Sodium borohydride (38 mg, 1 mmole) was added to a solution of the aminovanillin (2.86) (201 mg, 1 mmole) in 10% aqueous sodium hydroxide (20 ml), the solution was warmed to about 80°C for 5 minutes, cooled and analysed by t.l.c., which showed only starting material (R_f 0.5, CHCl_3) and no other products. The solution was heated under reflux for two hours, then left for 16 hours, acidified with acetic acid and the brown amorphous precipitate filtered off. This was analysed by t.l.c. (silica gel, CHCl_3) which showed it to be mainly a polymeric low mobility material.

3,4-Dimethoxy-6-nitrobenzaldehyde (2.87).- This was synthesised from veratraldehyde (2.21) (10 g, 60 mmole) according to the preparation of Fletscher¹²⁹ to give 6-nitroveratraldehyde (9.4 g, 74%), m.p. 130-131°C {lit.,¹²⁹ 132-133°C}, ν_{max} 1688, 1607, 1575, 1527, 1444, 1405, 1335, 1290, 1230, 1170, 1065, 1026, 987, 888, 798 and 740 cm^{-1} .

Part 3Synthesis of the Indole Hapten5-Chloro-6,7-dimethoxy-N-methyl-1H-
indol-3-yloxoacetic AcidSection ASynthesis of 5-Chloro-6,7-dimethoxy-N-methyl-1H-indole

Sporidesmin-A (1.1) can be regarded as comprising an indole moiety (1.36) and a dioxopiperazine ring moiety. The synthesis and coupling of these components permitted Kishi *et al.* to complete their total synthesis of sporidesmin-A (page 18). In the present work, a somewhat different synthetic approach to sporidesmin-A was envisaged, which will be described in part 4, but this approach also required the synthesis of the indole moiety. Some time before the communication by Kishi *et al.* appeared, a program aimed at the synthesis of the wanted indole was initiated, and its successful completion, described in this section, means that there are now two independent preparations of 5-chloro-6,7-dimethoxy-N-methyl-1H-indole (1.36).

The approach was based on the previous benzenoid work in which an efficient preparation of 5-chloro-2-nitroveratraldehyde (2.15) was devised, and this O-nitrobenzaldehyde was transformed into the indole (3.3) by the methods of Julia *et al.*,¹¹⁹ with a methylation step completing the sequence. (Scheme 3a). Each reaction step was tested on at least one model compound before its application in this sequence, and each step, with the model reactions, will be discussed in turn.

Condensation of an aldehyde with nitromethane

The general indole synthesis which involves the condensation of an O-nitrobenzaldehyde with a nitromethane followed by reduction of the nitro groups has been reviewed by Sundberg¹⁴⁸ and rather more fully, by Brown.¹⁴⁹ The method¹¹⁹ was applied in the present work initially with the model system shown. (Scheme 3b).

The styrene (3.5) has been obtained previously¹⁵⁰ by heating the aldehyde (2.48) with nitromethane, acetic anhydride and ammonium acetate;

the yield, however, was only 32%. In a low temperature method, using alcoholic potassium hydroxide and nitromethane followed by acidification, De Antoni et al.¹⁵¹ obtained the styrene (3.5) as yellow flakes in a yield of 47%. Essentially the same method was used in this present work, but an intermediate oil was obtained which was found by PMR, to be the alcohol (3.4). Heating the oil in acetic anhydride and sodium acetate gave the styrene (3.5) as yellow needles in 65% overall yield from the aldehyde (2.48).

When the reaction was carried out on the chloroaldehyde (2.15) (Scheme 3a), the alcohol (3.1) was also obtained as an oil, which on heating in toluene with removal of the water-toluene azeotrope, gave crystals of the alcohol (3.1) on cooling. Generally, the alcohol was not crystallised but was converted directly to the styrene (3.2) in an overall yield of 94% from the aldehyde (2.15).

There is a considerable difference between the PMR coupling patterns of the alcohols (3.1 and 3.4). In the non-chlorinated case (3.4), the α -proton resonates as a triplet, the β -protons as a doublet, and there is no evidence of coupling with the hydroxyl proton, the resonance of which was not observable. The pattern then, is that of an A_2M spectrum. The β -protons need not be identical since the adjacent (α) centre is chiral. The chloroalcohol (3.1) has a more complex spectrum in which the α -proton couples with the hydroxyl proton ($J_{\alpha,OH} = 4$ Hz) and separately with each of the β -protons ($J_{\alpha,\beta 1} = 5$ Hz, $J_{\alpha,\beta 2} = 7$ Hz). The two β -protons have very close shifts ($\delta_{\beta 1} = 4.61$, $\delta_{\beta 2} = 4.59$ ppm) and in the absence of the vicinal proton, the resonances would be observed as a singlet since the shift difference (1.2 Hz) is considerably less than the typical geminal coupling constant ($J_{\beta 1,\beta 2} \approx 12$ Hz). The two protons are readily distinguished only because of the differences in the vicinal coupling

constant ($J_{\alpha\beta}$). Evidently for the non-chlorinated alcohol (3.4), the vicinal coupling constants are identical.

The PMR spectra of the styrenes (3.2 and 3.5) permit the configurations of the double bonds to be determined. The couplings, $J_{\alpha,\beta} = 13$ Hz for 3.2 and $J_{\alpha,\beta} = 14$ Hz for 3.5 are typical for trans-olefins. This stereochemical point has apparently not been discussed before in relation to such nitrostyrenes.

The reductive cyclisation of α,β -dinitrostyrenes to indoles is discussed in the reviews of Sundberg^{148a} and Brown,^{149a} and this method of preparing indoles has been used particularly in the synthesis of alkoxyindoles, because of the ease of obtaining the styrene precursors from 2- and 6-nitrovanillin derivatives. Once more, the non-chlorinated system was used to develop preparative methods. Benington *et al.*¹⁵⁰ had obtained the indole (3.7) in 23% yield by catalytic hydrogenation of the styrene (3.5) and De Antoni *et al.*¹⁵¹ obtained the same indole in 55% yield by reduction with iron and acetic acid in ethanol. In the present work, the use of iron and aqueous acetic acid (according to Julia *et al.*,¹¹⁹) gave the indole (3.7) in 74% yield. (Scheme 3c).

Scheme 3c

However, this method was less successful in the preparation of the chloroindole (3.3), which was obtained only in 35% yield. No generally

suitable reducing agent has been found,^{149a,152} particularly for large scale reactions, and a better method would be desirable. No mechanism has been firmly established for the reaction,^{149a} but a diamine intermediate such as 3.6 which can ring close with loss of ammonia to give the indole, has been suggested as a possibility.¹⁵³

The PMR spectra of the indoles (3.3 and 3.7) were in accord with the proposed structures. For both compounds the coupling constants ($J_{1,2} = J_{1,3} = 2 \text{ Hz}$, $J_{2,3} = 3 \text{ Hz}$) were similar to those found for indole ($J_{1,2} = 2.5 \text{ Hz}$, $J_{1,3} = 2.0 \text{ Hz}$, $J_{2,3} = 3.1 \text{ Hz}$),¹⁵⁴ except that the coupling of H^1 and H^4 observed for indole ($J_{1,4} = 0.8 \text{ Hz}$) was not observed for the dimethoxy compounds.

The alcohol (3.1) could be reduced as for the styrene and cyclised to give the indole (3.3) without the isolation of the intermediate styrene (3.2). Yields were lower (ca. 20%)

Indole, 3.8

than for the styrene reduction (35%), and the major portion of the reaction products was not isolated, possibly because it comprised amines soluble in dilute acetic acid. A small amount of an indigo derivative (3.10) was isolated, which may have resulted from the dimerisation of the indoxyl (3.9).^{148b} (Scheme 3d).

Scheme 3d

The blue indigo (3.10) was readily and reversibly reduced to the light yellow leucoindigo (3.11) by glucose and base^{155a} and, as the conditions of the reaction that produced the indigo (3.10) were highly reducing, the blue indigo isolated presumably resulted from air oxidation of the leucoindigo during extraction and isolation. The N,N'-dimethyl derivative of 3.10 has been found among the degradation products of sporidesmin-A.¹⁸

Methylation of the indole (3.3) was carried out by the procedure of Potts and Saxton.¹⁵⁶ Their preparation of N-methylindole (3.12) which is a useful model compound, was repeated with satisfactory results. The procedure uses the *in situ* generation of sodium amide; a strong base which reacts with indole to give the indolyl anion (3.13) in equilibrium with the metalated indole (3.14). Treatment of 3.13 with an alkylating agent gives the 1-alkyl derivative, but the metalated indole (3.14) gives 1- and 3-alkyl derivatives.^{148c,157} Highly polar solvents such as ammonia or dimethylformamide favour the formation of the ion (3.13) and are thus the preferred medium for the reaction. (Scheme 3e)

Scheme 3e

The dimethoxyindole (3.7) was methylated to give 6,7-dimethoxy-N-methyl-1H-indole (3.16), an oil which gave satisfactory analytical and

spectra data. Methylation of the chloroindole (3.3) also proceeded well to give the desired product (1.36),

provided that care was taken to ensure that sodium amide formation was complete. If sodium was still present, dechlorination took place, as well as methylation, to give a mixture of the indoles

(3.3 and 3.7) and their N-methylated derivatives, (1.36 and 3.16). The indole (1.36) melted some 5°C higher than the literature value,⁷⁰ but had satisfactory analytical and spectral data.

5-Chloro-6,7-dimethoxy-N-methyl-1H-indole (1.36) has been synthesised previously by a different route.⁷⁰ 2-Nitroveratraldehyde (2.48) was transformed into 6,7-dimethoxyoxindole (1.35), and thence to the indole (1.36) (Scheme 3f).

A comparative evaluation of the merits of the two syntheses of the indole (1.36), that of Kishi *et al.*⁷⁰ and the one presented in this work, is difficult because of the large number of steps between the final product (1.36) and their common starting material, vanillin (2.3).

As described in part 2, the veratraldehydes (2.15 and 2.48) can both easily be synthesised, and a comparison of the two routes from those two intermediates shows that the literature synthesis⁷⁰ has eight steps, whereas the synthesis described in this work has only four. No overall yield can be calculated for the literature synthesis as the yield of the oxindole (1.35) is not given,^{70,158} but the overall yield of 1.36 (from 2.15) in the present work was 27%. However, the indole formation step is the major contributor to this inefficiency, and should an improvement in the yield of the indole (3.3) be achieved, the method described in this work would be the superior method.

5-Chloro-1H-indole-2-carboxylic acid (3.19) was synthesised as a hydrophilic cross-hapten for antibody study by the Japp-Klingmann method^{148d} and had satisfactory spectral and physical data. (Scheme 3g).

Scheme 3g

Section BSynthesis of the Indole Hapten

A hapten based on the indole (1.36) was required for several reasons. As the degree of synthetic elaboration increases, so will the cost of any derived vaccine, so that a balance must be struck between a small molecule bearing little resemblance to the sporidesmins, and a close analogue that requires many steps for its synthesis. Since "resemblance" ultimately is judged by an unpredictable conformation of amino acids in an antibody, the testing of fragments of the toxin as haptens is necessary in order to evaluate their ability to generate antibodies specific to the sporidesmins. The indole moiety (1.36) is the largest fragment common to all the sporidesmins, and as it bears a closer resemblance than the hapten (2.1) to the sporidesmins, it would appear to be a suitable basis for the synthesis of a hapten.

In order to couple the indole (1.36) to a protein, an intermediate spacer group is required with the following specifications:

- 1) The group should be bonded at the 2- or 3-position so as to leave the resemblance of the indole to sporidesmin unaltered. It is further preferable that the group possess some of the characteristics of the non-indole portion of sporidesmin-A, such as hydrophilicity.
- 2) The group should be capable of being coupled to a protein.
- 3) The hapten, when coupled to a protein, should possess a chromophore absorbing at wavelengths longer than 280 nm. (See page 32).

Oxalyl chloride is a reagent that reacts with indoles in high yield specifically at the 3-position, to give monomeric products capable of reacting further with alcohols, amines and water. Trial studies on the unsubstituted model, N-methylindole (3.12) showed that the acid resulting

from hydrolysis of the acyl chloride (1.37) might be a suitable hapten. The communication by Kishi *et al.*⁷⁰ gave little experimental detail for the preparation of 1.37, but treatment of the indole (1.36) with oxalyl chloride according to Buzas *et al.*¹⁵⁹ gave a crystalline derivative, presumed to be 1.37, which was not isolated but was hydrolysed to give the acid (3.20). (Scheme 3h). The ester (3.21) was obtained by treating the acyl chloride (1.37) with absolute alcohol. There is no adequate

Scheme 3h

explanation known for the production, in the presence of an excess of the indole (1.36), of the monomeric acyl chloride (1.37) rather than the dimeric product in which both acyl chloride groups have reacted with the indole. The precipitation of the product during the reaction helps prevent dimerisation, but as ethanol very rapidly reacts with the precipitate, a lack of reactivity of the indole (1.36) towards the chloride (1.37) is a necessary assumption. This lack of reactivity is so marked that the usual order of adding the reagents, namely oxalyl chloride to the indole, is the reverse to that required to minimise dimer formation. The acyl chloride does not appear to be under any severe steric restriction on

further reaction, and freely does react with a number of nucleophiles, so that no ready answer is apparent.

Indoles have ultraviolet spectra¹⁶⁰ rather similar to many proteins, which is explained by the presence in many proteins of the amino acid, tryptophan - an indole derivative. However, the extended conjugation resulting from substitution with oxalyl chloride gives rise to a red shift in some of the transitions, and the acid (3.20) was observed to have a strong absorbance at 337 nm ($\log \epsilon = 4.02$). This is satisfactory for establishing the presence of the hapten on a protein.

Kishi et al.⁷⁰ had shown that oxalyl chloride reacted with the indole (1.36) in high yield to give the acyl chloride (1.37). This work was repeated on the model, *N*-methylindole (3.12), with the intention of treating the resulting acyl chloride with *p*-phenylenediamine, to give the compound (3.25) which could be azo-linked to a protein. (Scheme 2i). Preparation of the aniline derivative (3.25) was found to be difficult, chiefly because *p*-phenylenediamine is soluble in only very polar solvents, and the work was abandoned when it was found that the acid (3.23) and its ethyl ester (which modelled the coupled hapten) possessed chromophores meeting the hapten requirements.

Scheme 2i

An attempt was made to reduce the keto-carbonyl group of the acid (3.23) to give an α -hydroxy acid which would possess an hydroxyl group at a similar position to one in sporidesmin-A (1.1). The keto-carbonyl was not reduced by sodium borohydride, the only result of the attempt being to precipitate the rather insoluble sodium salt of the acid (3.23).

The corresponding oxoacetic acid (3.26) of the non-chlorinated dimethoxyindole (3.7) was also prepared for use in cross hapten studies.

The H-2 and H-4 magnetic resonances

of indoles are sensitive to substitution

in the 3-position. The oxoacetic acid derivatives

show a 1.5 to 2 ppm downfield shift for the H-2

resonances and approximately half that effect for the H-4 resonances.

The effects are explicable in terms of a through space effect of the two carbonyls^{135b} resulting from confor-

mations such as 3.27. The effects on both

H-2 and H-4 confirm that 3-substitution

has taken place. This is a useful check

that 1-substitution rather than 3-substitution took place during

methylation, since a 3-methylindole cannot give an oxoacetic acid

derivative which has both the H-2 and H-4 protons shifted downfield.

Section CExperimental

General statements regarding spectroscopic, analytical and physical data will be found on page 85. The PMR spectra, however, are included with the experimental details in this section, with an asterisk in parentheses implying that the shift values are not corrected for paper expansion.

1H-Indole (3.8).- This compound was obtained commercially from the Sigma Chemical Company, and was used without further purification. It had m.p. 51.5-52°C {lit., ^{155b} 52°C}, $\nu_{\max}$ 3400, 1421, 1358, 1342, 1246, 1091, 1060, 936 747 and 726 cm^{-1} , PMR (CCl_4 , *), 7.40 (1H, m, H-4), 7.00-6.70 (3H, m, H-5,6,7) 6.48 (1H, q, $J_{2,3} = 3 \text{ Hz}$, $J_{1,2} = 2.5 \text{ Hz}$, H-2), 6.22 (1H, q, $J_{2,3} = 3 \text{ Hz}$, $J_{1,3} = 2 \text{ Hz}$, H-3).

3-Chloro-4,5-dimethoxy-6, β -dinitrostyrene (3.2).- 5-Chloro-2-nitroveratraldehyde (2.15) (12.3 g, 50 mmole) was suspended in ethanol (150 ml) and nitromethane (4.2 g, 71 mmole) and cooled to -15°C. Potassium hydroxide (12 g, 0.2 mole) in water (16 ml) and ethanol (150 ml) was added dropwise over 2 hours to the slurry stirred in an ice-bath. The slurry, was then acidified with concentrated hydrochloric acid (30 ml, 0.3 mole), diluted with water (200 ml) and extracted three times with chloroform (50 ml). The combined extracts were dried with sodium sulphate, and evaporated under vacuum to an oil. The oil was heated under reflux in acetic anhydride (50 ml, 0.5 mole) and sodium acetate (5 g) for 30 minutes, then poured into water (200 ml), stirred for 10 minutes, and the yellow-brown precipitate filtered off and dried under vacuum to give the styrene (3.2) (13.6 g, 94%). Recrystallised from ethanol (400 ml), it had m.p. 148-149°C, $\nu_{\max}$ 1640, 1535, 1356,

1300, 1028, 960, 910, 850 and 805 cm^{-1} , PMR (d^5 -pyridine) 8.12 (1H,d, $J=13.3$ Hz, H- β), 7.76 (1H,d,H- α), 7.70 (1H,s,ArH), 3.72 (3H,s,OMe), 3.69 (3H,s,OMe).

(Found C, 42.0; H, 3.0; Cl, 12.7; N, 9.6; $\text{C}_{10}\text{H}_9\text{ClN}_2\text{O}_6$ requires C, 41.6; H, 3.1; Cl, 12.3; N, 9.7%).

1-(3-chloro-4,5-dimethoxy-6-nitrophenyl)-2-nitroethanol (3.1).-

A sample of the oil from the above reaction was heated under reflux in toluene, with water removal, cooled and crystals that formed were filtered off to give the nitroalcohol (3.1). Recrystallised twice from benzene, it had m.p. 104-105°C, ν_{max} 3450, 1595, 1569, 1531, 1426, 1360, 1287, 1105, 1061, 1018, 959, 900, 870 and 801 cm^{-1} , PMR (CDCl_3) 7.46 (1H,s,ArH), 5.74 (1H,m,H- α), 4.61 (1H,d, $J_{\alpha,\beta}=5$ Hz, H- β 1), 4.59 (1H,d, $J_{\alpha,\beta}=7$ Hz, H- β 2), 3.99 (3H,s,OMe), 3.94 (3H,s,OMe), 3.32 (1H,d, $J_{\alpha,\text{OH}}=4$ Hz, OH) (Found, C, 39.4; H, 3.4; Cl, 11.8; N, 9.3; $\text{C}_{10}\text{H}_{11}\text{ClN}_2\text{O}_7$ requires C, 39.2; H, 3.6; Cl, 11.6; N, 9.1%).

5-Chloro-6,7-dimethoxy-1H-indole (3.3).- A stirred mixture of acetic acid (40 ml) and water (10 ml) was warmed to 50°C, and the styrene (3.2) (5.77 g, 20 mmole) was added, followed by iron powder (BDH Chemicals, hydrogen reduced) (2 g, 36 mmole). The stirred mixture increased in temperature until reflux began. When reflux had ceased, iron powder (10 g, 180 mmole) and acetic acid (30 ml) were added in portions to keep the mixture hot and fluid, and, after the addition was complete, stirring was continued for a further 30 minutes. The mixture was then diluted with water (200 ml) and extracted three times with chloroform (20 ml), the combined extracts were washed with sodium bicarbonate solution, dried with sodium sulphate and evaporated under vacuum to an oil. The oil was extracted with hot petroleum ether (10 ml) three times, and the combined extracts cooled to give the indole (3.3) (1.47 g, 35%).

Recrystallised from petroleum ether, the white crystals had m.p. 73-75°C, $\nu_{\max}$ 3320, 1568, 1450, 1302, 1205, 1047, 972, 835, 820 and 747 cm^{-1} , PMR (CCl_4) 8.45 (1H, bm, NH), 7.23 (1H, s, H-4), 6.94 (1H, q, $J_{1,2}=2$ Hz, $J_{2,3}=3$ Hz, H-2), 6.26 (1H, q, $J_{1,3}=2$ Hz, $J_{2,3}=3$ Hz, H-3), 3.94 (3H, s, OMe), 3.84 (3H, s, OMe). (Found, C, 57.0; H, 4.7; Cl, 16.9; N, 6.5; $\text{C}_{10}\text{H}_{10}\text{ClNO}_2$ requires C, 56.7; H, 4.8; Cl, 16.8; N, 6.6%).

5-Chloro-6,7-dimethoxy-N-methyl-1H-indole (1.36).- The indole (3.3) (1.69 g, 8 mmole) was methylated according to Potts and Saxton¹⁵⁶ to give a brown oil which was separated by preparative t.l.c. into a number of compounds (silica gel, CHCl_3) of which only the most mobile was collected. The product was eluted from the silica gel by 10% methanol in chloroform, and crystallised from methanol to give the N-methylindole (1.36) (1.46 g, 81%). Recrystallised from petroleum ether, it had m.p. 50-50.5°C {lit.,⁷⁰ 44-45°C}, $\nu_{\max}$ 1559, 1480, 1410, 1296, 1235, 1045, 981, 838 and 742 cm^{-1} , PMR (CDCl_3) 7.38 (1H, s, H-4), 6.92 (1H, d, $J=3.0$ Hz, H-2), 6.35 (1H, d, $J=3.0$ Hz, H-3), 4.01, 3.94 and 3.92, each (3H, s, Me). (Found, C, 58.5; H, 5.3; Cl, 16.0; N, 6.0; $\text{C}_{11}\text{H}_{12}\text{ClNO}_2$ requires C, 58.5; H, 5.4; Cl, 15.7; N, 6.2%).

When methylation of the indole (3.3) was carried out according to Potts and Saxton,¹⁵⁶ except that the ferric nitrate nonahydrate catalyst was not added to aid dissolution of the sodium in the ammonia, a mixture of four products was obtained that were separated by analytical t.l.c. (silica gel, CHCl_3 elutant, 1% H_2SO_4 in ethanol spray followed by heating to give purple and crimson spots). The products were found to be 5-chloro-6,7-dimethoxy-N-methyl-1H-indole (1.36) (R_f 0.8), 6,7-dimethoxy-N-methyl-1H-indole (3.16) (R_f 0.7), starting material (3.3) (R_f 0.4) and 6,7-dimethoxy-1H-indole (3.7) (R_f 0.3), in approximately equal amounts of 1.36 and 3.7, and somewhat less of the other two products, 3.3 and 3.16.

3,4-Dimethoxy-2,β-dinitrostyrene (3.5). - 2-Nitroveratraldehyde

(10.56 g, 50 mmole) (page 97) and treated with nitromethane and base as for the preparation of the chlorostyrene (3.2), to give as an oil, 1-(3,4-dimethoxy-2-nitrophenyl)-2-nitroethanol (3.4) (13.0 g, 96%). PMR (CDCl₃) 7.34 (1H,d,J=8.5 Hz, ArH), 7.11(1H,d,J=8.5 Hz, ArH), 5.90 (1H,t,J=6 Hz, H-α), 4.62 (2H,d,J=6 Hz, H-β), 3.92 (3H,s,OMe), 3.92 (3H,s,OMe), OH not observed. The oil was heated under reflux in acetic anhydride and sodium acetate as for the chlorobenzylalcohol (3.1) to give a fluffy precipitate of the styrene on dilution with water. Recrystallised from chloroform, the yellow needles of the styrene (8.34 g, 65%) had m.p. 165-167°C {lit.,¹⁵¹ 169-170°C}, $\nu_{\max}$ 1635, 1609, 1348, 1290, 1055, 955, 820 and 800 cm⁻¹, PMR (d⁶-DMSO,*) 7.84 (1H,d,J=14 Hz, H-α), 7.65 (1H,d,J=9 Hz,ArH), 7.49 (1H,d,J=14 Hz,H-β), 7.16 (1H,d,J=9 Hz,ArH), 3.96 (3H,s,OMe) and 3.85 (3H,s,OMe).

6,7-Dimethoxy-1H-indole (3.7). - The styrene (3.5) (2.54 g,

10 mmole) was reduced with iron powder and acetic acid as for the chlorostyrene (3.1) and the products were separated by preparative t.l.c. with only the fastest component being collected. This gave white crystals of the indole (3.7) (1.31 g, 74%). Recrystallised from methanol, it had m.p. 101-102°C {lit.,¹⁵⁰ 102-103°C}, and a sublimed sample had $\nu_{\max}$ 3400, 3330, 1626, 1578, 1509, 1450, 1338, 1251, 1225, 1083, 1051, 975, 880 and 800 cm⁻¹, PMR (CDCl₃) 8.50 (1H,bm,NH), 7.38 (1H,d,J=8.5 Hz, ArH), 7.11 (1H,q,J_{1,2}=2.4 Hz, J_{2,3}=3 Hz, H-2), 6.92 (1H,d,J=8.5 Hz, ArH), 6.53 (1H,q,J_{1,3}=2.4 Hz, J_{2,3}=3 Hz, H-3), 4.03 (3H,s,OMe) and 3.94 (3H,s,OMe).

2-(5-Chloro-1,3-dihydro-6,7-dimethoxy-N-methyl-3-oxo-2H-indol-2-ylidene)-5-chloro-1,2-dihydro-6,7-dimethoxy-N-methyl-3H-indol-3-one (3.10) (an indigo derivative). - The chlorobenzyl alcohol (3.1) (18.3 g, 60 mmole) was reduced with iron powder and acetic acid in several batches, as for the styrene (3.2), to give blue oils, which were largely dissolved in ether

leaving a precipitated blue solid. The ether soluble fractions were found to consist largely of the indole (3.3) in yields of 10 to 30%. The various samples of blue precipitate were combined and recrystallised from toluene to give the indigo (3.10) (61 mg, 0.2%), which had m.p. ca. 275°C, $\nu_{\max}$ 1645, 1615, 1583, 1488, 1452, 1442, 1420, 1388, 1315, 1150, 1117, 1037, 976, 833 and 663 cm^{-1} , insoluble in usual PMR solvents. (Found C, 53.0; H, 3.5; Cl, 15.9; N, 6.4; $\text{C}_{20}\text{H}_{16}\text{N}_2\text{O}_6$ requires C, 53.2; H, 3.6; Cl, 15.7; N, 6.2%).

On heating a small sample in 10% sodium hydroxide with glucose, the blue indigo solid dissolved to form a light yellow solution, which on acidification, warming and shaking in air, precipitated a blue solid.

• N-Methyl-1H-indole (3.12).- Indole (3.8) (23.4 g, 0.20 mole) was methylated according to Potts and Saxton¹⁵⁶ gave N-methylindole (3.12) in 96% yield. Distillation (110°C, ca. 15 mm Hg) failed to remove a small amount (2-5%) of indole. A frozen sample melted 3-5.5°C and the liquid had n_D^{22} 1.6058 {lit.,¹⁵⁶ $n_D^{18.5}$ 1.6082}, $\nu_{\max}$ (neat) 3020, 2900, 1615, 1521, 1474, 1337, 1324, 1242, 1078, 1014, 765 and 745 cm^{-1} , PMR (CCl_4 , *) 7.45 (1H,m,H-4, 7.1-6.8 (3H,m,H-5,6,7), 6.56 (1H,d, J=3.1 Hz,H-2), 6.26 (1H,d,J=3.1 Hz, H-3) 3.22 (3H,s,NMe).

6,7-Dimethoxy-N-methyl-1H-indole (3.16).- The dimethoxyindole (3.7) (1.06 g, 6 mmole) was methylated according to Potts and Saxton¹⁵⁶ to give the N-methylindole (3.16) as an oil, which was purified by preparative t.l.c. to give a clear colourless oil (0.963 g, 84%), $\nu_{\max}$ (neat) 2940, 2830, 1625, 1509, 1465, 1310, 1267, 1077, 975, 798 and 703 cm^{-1} , PMR (CDCl_3 , *) 7.03 (1H,d,J=8 Hz, ArH), 6.56 (1H,d,J=8 Hz, ArH), 6.51 (1H,d,J=3Hz, H-2), 6.17 (1H,d,J=3 Hz,H-3), 3.72, 3.65 and 3.65, each (3H,s, Me). (Found, C, 68.8; H, 6.9; N, 7.0; $\text{C}_{11}\text{H}_{13}\text{NO}_2$ requires C, 69.1; H, 6.9; N, 7.3%).

5-Chloro-1H-indol-2-ylcarboxylic acid (3.19).— p-Chloroaniline

(3.2 g, 25 mmole) was converted into ethyl 5-chloro-1H-indol-2-carboxylate by the method of Heath-Brown and Philpott¹⁶¹ and this product was hydrolysed according to Rydon and Tweedle¹⁶² to give the acid (3.19) (1.2 g, 25%). Recrystallised from 50% aqueous ethanol, it had m.p. 287–289°C [lit.,²⁰¹ 289–290°C], $\nu_{\max}$ 3410, 1660, 1543, 1331, 1295, 1259, 1060, 875 and 764 cm^{-1} , PMR (NaOD/D₂O; no internal standard, highest field signal arbitrarily set at 7.0 ppm) 7.62 (1H, d, $J_{4,6}$ = 2 Hz, H-4), 7.40 (1H, d, $J_{6,7}$ = 8.8 Hz, H-7), 7.18 (1H, q, $J_{6,7}$ = 8.8 Hz, $J_{4,6}$ = 2 Hz, H-6), 7.00 (1H, s, H-2).

5-Chloro-6,7-dimethoxy-N-methyl-1H-indole-3-yloxoacetic acid

(3.20).— The indole (1.36) (0.903 g, 4 mmole) was dissolved in anhydrous ether (20 ml) and cooled to –20°C. Oxalyl chloride (0.5 ml, 5.8 mmole) was added dropwise to the stirred solution over a minute, during which a precipitate of the acyl chloride (1.37) formed, and after 5 minutes, sodium hydroxide (0.40 g, 10 mmole) in water (10 ml) was added and stirring was continued for a further 10 minutes. A yellow precipitate was filtered off from the acidic solution and dried under vacuum to give the acid (3.20) (1.024 g, 86%). Recrystallised twice from benzene, the yellow plates had m.p. 185–187°C, $\nu_{\max}$ 3290, 3120, 1744, 1400, 1352, 1310, 1270, 1127, 1028, 969, 865 and 829 cm^{-1} , PMR (d^6 -DMSO, *) 11.2 (1H, bs, COOH), 8.35 (1H, s, H-2), 7.90 (1H, s, H-4), 4.04, 3.97 and 3.85, each (3H, s, Me), UV, $\nu_{\max}$ (log ϵ) 337 nm (4.02), 281 nm (3.9) and 263 nm (4.00), (in 0.1 M HCl in methanol). (Found C, 52.8; H, 4.0; Cl, 12.3; N, 4.8; C₁₃H₁₂ClNO₅ requires C, 52.6; H, 4.1; Cl, 11.9; N, 4.7%).

Ethyl 5-chloro-6,7-dimethoxy-N-methyl-1H-indol-3-yloxoacetate

(3.21).- The indole (1.36) (135 mg, 0.6 mmole) was treated with oxalyl chloride (0.064 ml, 0.75 mmole) as above to give the acyl chloride (1.37) to which was added dry ethyl alcohol (0.5 ml, 8.57 mmole). The solution was left for one minute and then poured into water (20 ml) saturated with sodium bicarbonate. The product was extracted twice with ether (10 ml), the combined ether extracts were washed with water, dried with sodium sulphate and vacuum evaporated to an oil which crystallised to give the ester (3.21) (150 mg, 77%). Recrystallised from petroleum ether, it had m.p. 117-118°C, $\nu_{\max}$ 3330, 3150, 1776, 1616, 1406, 1330, 1302, 1267, 1122, 1087, 962, 911 and 793 cm^{-1} , PMR (CDCl_3) 8.17 (1H,s,H-2), 8.17 (1H,s,H-4), 4.38 (2H,q,J=7 Hz, CH_2), 4.02, 4.02 and 3.90, each (3H,s,Me), 1.42 (3H,t, J=7 Hz, CH_3). (Found, C, 55.1; H, 4.9; Cl, 11.2; N, 4.0; $\text{C}_{15}\text{H}_{14}\text{ClNO}_5$ requires C, 55.3; H, 5.0; Cl, 10.9; N, 4.3%).

N-Methyl-1H-indol-3-yloxoacetic acid (3.23).- N-Methylindole

(1.31 g, 10 mmole) was treated with oxalyl chloride (1.0 ml, 10.7 mmole) as for the preparation of the acid (3.20) to give after hydrolysis of the chloride, the acid (3.23) (1.58 g, 78%). Recrystallised from chloroform-petroleum ether, it had m.p. 155-158°C, {lit.,¹⁶³ 160-162°C}, $\nu_{\max}$ 3265, 3150, 1759, 1621, 1525, 1403, 1320, 1217, 1086, 907, 752 and 743 cm^{-1} , PMR (CDCl_3 - d^6 DMSO), 8.50 (1H,s,H-2), 8.42 (1H,m,H-4), 8.35 (1H,s,COOH), 7.50-7.20 (3H,m,H-5,6,7), 3.86 (3H,s,NMe).

Ethyl N-methyl-1H-indol-3-yloxoacetate (3.24).- N-Methylindole

(6.56 g, 50 mmole) was treated with oxalyl chloride (7 ml, 75 mmole) over 5 minutes, as for the acid (3.20), left to warm to room temperature, and the supernatant ether decanted from the precipitated acyl chloride (3.22). Absolute alcohol (20 ml, 350 mmole) was added, then triethylamine and the mixture filtered, evaporated under vacuum to give the ester (3.24).

Recrystallised from alcohol, the ester (4.2 g, 36%) had m.p. 88-90°C {lit., ¹⁶⁴ 90-91°C}, $\nu_{\max}$ 1722, 1635, 1530, 1474, 1278, 1193, 1083, 936 and 750 cm^{-1} , {lit., ¹⁶⁴ 1720, 1630 cm^{-1} }, PMR (CDCl_3 , *) 8.31 (1H,m, H-4), 8.11 (1H,s,H-2), 7.30-7.15 (3H,m,H-5,6,7), 4.35 (2H,q,J=7 Hz, CH_2), 3.73 (3H,s,NMe), 1.40 (3H,t,J=7 Hz, CH_3), UV, $\nu_{\max}$ (log ϵ), 327 nm (4.04), 276 nm (4.00), 260 nm (4.09) (in methanol).

Attempted borohydride reduction of N-methylindol-3-yloxoacetic acid (3.23).— The acid (102 mg, 0.5 mmole) was dissolved in 10% sodium hydroxide (5 ml) by warming, sodium borohydride (19 mg, 0.5 mmole) was added, the solution was heated close to boiling then left to cool for two hours, and the crystals which formed were filtered off and dried. Infrared analysis showed the product was the sodium salt of the acid (3.23), which could be easily obtained by dissolving the acid (3.23) in warm 10% sodium hydroxide and cooling. The sodium salt had $\nu_{\max}$ (hexachlorobutadiene) 3250, 1488, 1478, (nujol) 1617, 1528, 1209, 1122, 1085 and 739 cm^{-1} , UV, $\nu_{\max}$ (log ϵ), 317 nm (4.07) and 225 nm (4.10) (0.1 M NaOH in methanol)

6,7-Dimethoxy-N-methyl-1H-indol-3-yloxoacetic acid (3.26).— The indole (3.7) (1.91 g, 10 mmole) was treated with oxalyl chloride (1 ml, 11.7 mmole) as for the preparation of the acid (3.20) to give the acid (3.26) (2.29 g, 87%). Recrystallised from benzene, it had m.p. 145-150°C, $\nu_{\max}$ 3325, 3140, 1776, 1615, 1405, 1329, 1302, 1267, 1123, 1086, 962, 910 and 794 cm^{-1} , PMR (CDCl_3 -d⁶DMSO, *) 8.13 (1H,s,H-2), 7.83 (1H,d, J=8.5,H-4), 6.89 (1H,d,H-5), 5.84 (1H,s,COOH), 3.98, 3.87 and 3.85, each (3H,s,Me). (Found, C, 59.6; H, 4.9; N, 5.2; $\text{C}_{13}\text{H}_{13}\text{NO}_5$ requires C, 59.3; H, 5.0; N, 5.3%).

Part 4 Elaboration of the Indole Nucleus Towards Pyrroloindoles

Section A

Synthesis and reactions of

1-methyl-3-(N,N-dimethylimonio)methyl-1H-indole chloride

This section of the work had the aim of making the pyrroloindole derivative (4.5) as a precursor to sporidesmin-A and haptens closely related to it. This precursor could possibly be synthesised by the reaction sequence as in scheme 4a. There is a need for a 3-disubstituted methylindole, such as the dibromide (4.2), which is capable of monodisplacement by the N-acetamidomalonate ion derived from 4.3 to give the compound (4.4). Under the base conditions of the displacement reaction, this could then undergo a ring closure reaction to give the desired precursor, which has a double bond suitable for hydroxylation. The hapten (4.7) would then be accessible as shown (Scheme 4a).

The reaction sequence leading to the indole (1.36) has been described in part 3. None of the steps in scheme 4a has been carried out on the fully substituted indole (1.36), but model studies have been carried out using the readily obtained compound, *N*-methylindole (3.12). As the 3-formyl derivative would be the starting material for obtaining the required 3-disubstituted methylindole, 3-formyl-*N*-methylindole was prepared by two different routes (Scheme 4b).^{165,166}

Scheme 4b

Indole (3.8) and *N*-methylindole (3.12) were each formylated by the Vilsmeier-Haack reaction, the many and varied forms of which have recently been reviewed.¹⁶⁷ The reaction, in this case, involves the use of dimethylformamide (DMF) and phosphoryl chloride ($POCl_3$), which react to give the intermediate electrophile (4.10), and this with indole (3.8) gives an intermediate 3*H*-indolidene (4.11) which can be hydrolysed in base to afford the 3-formyl derivative (4.8) (Scheme 4c).

Scheme 4c

The 3H-indolidene has been isolated previously by chloroform extraction of the reaction solution,¹⁶⁸ but the corresponding intermediate in the N-methylated reaction has not. Since the methyl on the nitrogen prevents 3H-indolidene formation, the intermediate (4.12) is quite different in character from the 3H-indolidene (4.11), and was isolated by filtration. It was insoluble in all aprotic solvents tested and was found to be soluble only in water and methanol. Recrystallisation was achieved by dissolving it in water and pouring the solution into acetone, from which the compound crystallised over a period of 5 minutes.

The PMR spectra of the formylindoles (4.8 and 4.9) unambiguously proved that substitution had occurred in the 3-position. This was established by comparison of the spectra with those of the unformylated compounds (3.8 and 3.12), and it was observed that the protons at C-2 and C-4 were deshielded in much the same manner as had been observed for the 3-oxoacetates (page 117), although the effect was not so pronounced. This deshielding was not observed in the PMR spectrum of the intermediate (4.12) which had the H-4 resonance occurring at the same shift (7.42) as the other aromatic protons, rather than 1 ppm downfield. The H-2 proton was downfield from the shift observed for N-methylindole (3.12) (δ 7.77 vs 6.57), but this can be ascribed to the change in solvent (D_2O vs CCl_4), since the H-2 shift is strongly dependent on solvent.¹⁵⁴

It is doubtful, therefore, if the suggested structure for the intermediate, that of the immonium salt (4.12), is an accurate representation of the compound. It may exist as the covalent compound (4.13) or as one of the canonical forms in scheme 4d. The compound is a white crystalline solid that did not melt, but slowly decomposed on

heating above 200°C. It was readily soluble in water and methanol, and gave an immediate precipitate of silver chloride on addition of silver nitrate solution to its aqueous solution. On heating it with methyl iodide, the methiodide was not formed but instead, the less soluble iodide salt (4.14) was obtained, which could also be obtained by mixing methanolic solutions of the chloride salt (4.12) and sodium iodide (scheme 4c). This behaviour suggests that the compound (4.12),

Scheme 4e

is primarily ionic in nature, and as the structure (4.12) has been assumed to be the favoured canonical form, the intermediate has been named 1-methyl-3-(N,N-dimethylimonio)methyl-1H-indole chloride.

Isolation of the immonium salt (4.12) was carried out because it had been suggested¹⁶⁹ as a preferable alternative to the dibromide (4.2). The chlorine atom was found to be labile and a trimethylammonium analogue (4.15) was known to undergo the desired displacement¹⁷⁰ (Scheme 4f). When the immonium salt was treated with diethyl N-acetamido-

Scheme 4f

malonate and sodium ethoxide in ethanol, only the trivial product 3-formyl-N-methylindole (4.9) was obtained; from the hydrolysis of the unreacted salt with aqueous base during the extraction of the products. This hydrolysis occurred so easily that atmospheric moisture alone was sufficient to give the formyl compound as the sole product.

Although the immonium salt could not be methylated, and so lacked the trimethylamine leaving group, both the chloride and the dimethylamine group could be displaced by unsubstituted malonate esters. Dimethyl malonate with sodium methoxide in methanol when heated under reflux with the immonium salt (4.12) gave the condensed product (4.18) in 52% yield. The formylindole (4.9) did not condense under the same conditions. Diethylmalonate and sodium ethoxide in ethanol with the immonium salt gave the analogous product (4.20) on stirring for two

hours at room temperature (Scheme 4g). The success of these reactions

Scheme 4g

implies that not only could both groups be displaced, but that hydrolysis was not preventing the reaction. Since only 0.2% water in the highly hygroscopic solvent would completely hydrolyse the immonium salt at the concentrations used, great care was taken to dry the solvents before use, generally by distillation from the appropriate magnesium alkoxide.¹⁴⁰

The hydrolysis product, 3-formyl-N-methylindole (4.9) was also found not to undergo displacement and ring closure on standing in solution with the anion of diethyl N-acetamidomalonate for 24 hours, nor on heating under reflux for one hour. However, an attempt was made at condensation using titanium tetrachloride, which has been recently reported to be an effective promoter of Knoevenagel condensations.^{171,172} In the literature method, a 4 M solution of the titanium reagent in carbon-tetrachloride is added to tetrahydrofuran (THF), which precipitates a titanium-THF-chloride complex. The aldehyde and malonate are then added followed by a slow addition of pyridine, and the mixture is left stirring for up to 4 days. The method was used on the formylindole (4.9) with diethyl N-acetylmalonate (4.3) and the mixture was left stirring for 15 days, but no reaction occurred.

As it appeared that the ring closure reaction could not be accomplished, and that condensation required a hydrogen atom β to the dimethylamino group after the initial displacement (Scheme 4h), attention

Scheme 4h

now shifted to the preparation of condensed glycine derivatives. These derivatives (for example, 4.21) might possibly undergo a ring closure reaction in the manner achieved by Kishi et al. in the synthesis of Sporidesmin-A (page 18, Scheme 1s).

Hellmann and Piechota¹⁷³ have found that electron deficient aldehydes will condense partially with monoalkyl *N*-acetamidomalonates by decarboxylation to give serine derivatives. Their reaction of *p*-nitrobenzaldehyde with monomethyl *N*-acetamidomalonate (4.23) was repeated, and the serine derivative (4.24) was obtained in 60% yield (Scheme 4i).

Scheme 4i

The reaction times used by these authors are generally much longer than 16 hours, benzaldehyde, for instance, required 8 days, and 3-formylindole (4.8) did not react.¹⁷³ This reflects the decreasing electrophilic nature of the carbonyl group, as one proceeds from nitrobenzaldehyde to the electron rich 3-formylindole. 3-Formyl-N-methyl indole, on the basis of these observations, could also be expected to be unreactive, and after 6 weeks reaction time, only the starting materials were observed (PMR). The immonium salt (4.12) was also subjected to the same reaction, at room temperature for 24 hours and then heating under reflux for 1 hour, but no condensation occurred under either of these conditions. Since the immonium group could be regarded as equivalent to an electron deficient carbonyl group, this lack of reactivity is surprising, and possibly reflects the charge delocalisation of the immonium salt (Scheme 4d), with the carbonium ion canonical forms having only a minor contribution.

Kirby and Varley³⁸ have produced a suitable glycine derivative (4.27) in 20% yield by heating the aldehyde (4.25) with N-acetyl glycine in acetic anhydride and anhydrous potassium carbonate (Scheme 4j). Ethyl

Scheme 4j

N-acetylglycinate may also be expected to condense under similar conditions either with the salt (4.12), or with the non-deuterated analogue of 4.25, the formylindole (4.9). Ethyl N-acetylglycinate (4.29) was prepared from

the monoethyl malonate (4.28) in almost quantitative yield by decarboxylation at 140°C, but the condensation experiment has yet to be carried out (Scheme 4k).

Scheme 4k

If ring closure by the method of Kishi *et al.*⁷⁰ was successfully carried out on the glycine derivative (4.21), the product (4.30) would be obtained. The relevance of this reaction may be seen if the two model compounds, *N*-methylindole (3.12) and ethyl *N*-acetylglycinate (4.29) are replaced by the fully substituted indole (1.36) and a glycine-alanine dioxopiperazine (4.32), which could give as the equivalent of 4.30, the compound (4.3), which is rather closely allied to sporidesmin-A (1.1) (Scheme 4l).

In the course of the work on the immonium salt (4.12), attempts were made to reduce the compound to 1-methylgramine (4.37), which is a useful compound capable of undergoing a number of displacement reactions (as in scheme 4f). Although it can be readily obtained in good yield by a Mannich condensation of dimethylamine and formaldehyde with N-methylindole, the reduction would be an alternative synthesis with the added advantage that no 1-unsubstituted gramine would be present. (Indole is a contaminant of N-methylindole,¹⁶⁵ but its Vilsmeier intermediate (4.11) does not contaminate the immonium salt (4.12)).

Sodium borohydride in methanol failed to reduce the immonium salt, which, after 30 minutes, was recovered from solution. This result is in contrast to that of Klutchko et al.¹⁷⁵ who reduced an analogous compound (4.36) to the diazagramine (4.37) with potassium borohydride (Scheme 4m).

Scheme 4m

When sodium metal was added to a solution of the immonium salt (4.12) in methanol, reduction of the salt did not occur, and on addition of water prior to extraction of the products, the salt was hydrolysed to the formyl derivative (4.9).

Lithium aluminium hydride was partially successful in reducing the salt to 1-methylgramine (4.37), but the insolubility of the salt in aprotic solvents greatly hampered the reaction. On decomposition of the excess of lithium aluminium hydride with water, the remaining salt was hydrolysed to 3-formyl-N-methylindole (4.9). When this reaction was repeated but with heating of the reaction mixture under reflux, only the formylindole (4.9) and a complex mixture of biproducts was observed on analysis by t.l.c.

Scheme 4n

Section BExperimental

See page 85 for instrumental details.

Phosphoryl chloride (POCl_3).— This was obtained commercially from British Drug Houses (BDH) Ltd., and distilled before use.

Dimethylformamide (DMF).— This solvent was obtained commercially from Ajax Chemicals Ltd, and was not further purified, but was kept dry by standing over Linde 4A molecular sieve.

Diethylmalonate (4.19).— This liquid was obtained commercially from British Drug Houses (BDH) Ltd, and was used without further purification apart from drying with calcium sulphate. It had the PMR spectrum (CDCl_3) 4.11 (4H,q,J=7 Hz, OCH_2 -), 3.28 (2H,s,- COCH_2CO -), 1.24 (6H,t, CH_3).

p-Nitrobenzaldehyde (4.22).— This compound was obtained commercially from British Drug Houses (BDH) Ltd, and was used without further purification. It had m.p. 105.5-106°C {lit., ^{155d} 106°C}, ν_{max} 1710, 1608, 1547, 1352, 1291, 1202, 1105, 1006, 855, 823, 743 and 685 cm^{-1} , PMR (CDCl_3) 10.10 (1H,s,CHO), 8.34 (2H,d,J=8.5 Hz, H-3 and H-5), 8.07 (2H,d,H-2 and H-6).

Diethyl N-acetamidomalonate (4.3).— This was obtained from the Sigma Chemical Company and was used without further purification. The white crystals had m.p. 95-96°C {lit., ^{155c} 95-97°C}, ν_{max} 3330, 1760, 1740, 1630, 1535, 1445, 1270, 1235, 1171, 1025, 1014 and 868 cm^{-1} , PMR (CDCl_3) 6.90 (1H,bd,J=7 Hz, NH), 5.12 (1H,d,J=7 Hz, CH-N), 4.20 (4H,q,J=7 Hz, $-\text{OCH}_2-$), 2.06 (3H,s, CH_3CO -), 1.29 (6H,s,- CH_3).

Dimethylmalonate (4.17).- Diethylmalonate (4.19) (32.03 g, 0.2 mole) was heated under reflux in dry methanol (160 g, 5 mole) containing sodium methoxide (0.01 mole) for 12 hours, the solvent was evaporated under vacuum to give an oil which was mixed with dry methanol again (80 g, 2.5 mole) and sodium methoxide (0.01 mole) and heated under reflux for a further 12 hours after which the solvent was again removed under vacuum to give dimethylmalonate, PMR (CDCl_3 , *) 3.66 (6H,s,- CH_3), 3.33 (2H,s,- $\text{COCH}_2\text{CO-}$). The PMR of the intermediate oil showed ca. 10% ethyl ester remained, but no such resonances were observed in the final product.

Dimethyl N-acetamidomalonate (4.38).- Diethyl N-acetamidomalonate (4.31) (10.86 g, 0.050 mole) was heated for 2 hours under reflux in methanol (64 g, 2 mole) containing sodium methoxide (0.001 mole), the solvent was largely evaporated under vacuum, more methanol (48 g, 1.5 mole) was added, and heating under reflux continued for another 2 hours. The solvent was again largely distilled off under vacuum and the remainder (ca. 20 ml) was chilled to -20°C . The needles that formed were filtered off and recrystallised from methanol to give dimethyl N-acetamidomalonate (4.38) (6.02 g, 64%), which had m.p. $127-128^\circ\text{C}$ {lit., ¹⁷⁶ 128.5°C }, ν_{max} 3320, 1755, 1658, 1548, 1286, 1165, 977, 967, 886 and 696 cm^{-1} , PMR (CDCl_3) 6.8 (1H,bd,J=7 Hz, NH), 5.19 (1H,d,J=7 Hz, CH-N), 3.75 (6H,s, COOCH_3), 2.04 (3H,s, COCH_3).

Monomethyl N-acetamidomalonate (4.23).- The dimethyl ester (4.38) (3.78 g, 20 mmole) was hydrolysed with potassium hydroxide (1.12 g, 20 mmole) by the method of Hellman et al., ¹⁷⁷ to give the monomethyl ester (4.23) (2.16 g, 62%) with m.p. $128-129^\circ\text{C}$ (decarboxylation) {lit., ¹⁷⁷ $131.5-132^\circ\text{C}$ }, ν_{max} 3360, 1755, 1715, 1600, 1530, 1345, 1265, 1235, 1167, 1010, 967 and 806 cm^{-1} .

Monoethyl N-acetamidomalonate (4.28).— The diethyl ester (4.3) (6.52 g, 30 mmole) was hydrolysed by potassium hydroxide (1.68 g, 30 mmole) by the method of Hellmann et al.,¹⁷⁷ to give the monoethyl ester (4.38) (3.22 g, 57%) with m.p. 127°C (decarboxylation) {lit.,¹⁷⁷ 132-132.5°C}, $\nu_{\max}$ 3310, 1745, 1595, 1530, 1428, 1335, 1260, 1237, 1175, 1014 and 865 cm^{-1} , PMR ($\text{d}^6\text{DMSO}, *$) 8.45 (1H, d, $J=7.2$ Hz, NH), 5.6 (bs, COOH and HDO), 4.90 (1H, d, $J=7.2$ Hz, CH-N), 4.08 (2H, q, $J=7$ Hz, $-\text{OCH}_2-$), 1.89 (3H, s, COCH_3), 1.17 (3H, t, $J=7$ Hz, CH_3).

Ethyl N-acetylglycine (4.29).— Monoethyl N-acetamidomalonate (4.28) (1.42 g, 7.5 mmole) was gently heated to its melting point (ca. 130°C), when decarboxylation rapidly took place, then was left at 140°C for 15 minutes, cooled, and after some hours, ethyl N-acetylglycine (4.29) (1.08 g, 98.7%) crystallised. Recrystallised from benzene-petroleum ether, it had m.p. 42-46°C {lit.,¹⁷⁸ 44-46°C}, $\nu_{\max}$ 3330(b), 3090, 1755, 1660, 1565, 1420, 1313, 1205, 1195, 1127, 1101, 1040, 1029 and 715 cm^{-1} , PMR (CDCl_3) 6.95 (1H, bd, $J=7$ Hz, NH), 4.12 (2H, q, $J=7$ Hz, $-\text{OCH}_2-$), 3.91 (1H, d, $J=7$ Hz, CH-N), 2.00 (3H, s, COCH_3), 1.25 (3H, t, CH_3).

3-Formyl-1H-indole (4.8).— Indole (3.8) (5.86 g, 50 mmole) was formylated by the Vilsmeier Haack method according to James and Snyder¹⁶⁶ to give, after recrystallisation from aqueous ethanol, 3-formylindole (4.8) (5.52 g, 76%), with m.p. 192-195°C {lit.,¹⁶⁶ 196-197°C}, $\nu_{\max}$ 3150, 1636, 1615, 1575, 1528, 1398, 1241, 1128, 1083, 790, 768 and 760 cm^{-1} , PMR (d^6DMSO) 12.2 (1H, s, NH), 10.02 (1H, s, CHO), 8.30 (1H, s, H-2), 8.20 (1H, m, H-4), 7.7-7.2 (5H, m, H-5, 6, 7).

3-Formyl-N-methyl-1H-indole (4.9).— 3-Formylindole (4.8) (1.45 g, 10 mmole), methyl iodide (4.26 g, 30 mmole) and potassium carbonate (4 g, 29 mmole) in acetone (20 ml) were heated under reflux

for 6 hours, until t.l.c. analysis (silica gel, CHCl_3 , 5% H_2SO_4 in ethanol spray then heat to develop colour) showed that the starting material (R_f 0.15, orange) had been converted to the methylated product (4.9) (R_f 0.33, purple). The mixture was filtered, the residue was washed with acetone, the combined filtrate and washings were diluted with 0.2 M hydrochloric acid (200 ml), extracted three times with chloroform (10 ml), then the combined chloroform extracts were dried with sodium sulphate and evaporated under vacuum to give an oil which was mixed with ether (15 ml) and left to stand. The white prisms which slowly formed were filtered off to give 3-formyl-N-methylindole (1.11 g, 85%). The product was recrystallised from ether by soxhlet extraction to give white crystals with m.p. $69-70^\circ\text{C}$ {lit.,¹⁶⁵ 67°C }, ν_{max} 1640, 1540, 1385, 1335, 1184, 1121, 1071, 790, 746 and 727 cm^{-1} , PMR (CDCl_3 , *) 9.73 (1H, s, CHO), 8.16 (1H, m, H-4), 7.35 (1H, s, H-2), 7.25-7.05 (3H, m, H-5, 6, 7), 3.58 (3H, s, NMe).

3-Formyl-N-methylindole (4.8) (5.83 g, 61%) was also obtained from N-methylindole (3.12) (7.87 g, 6 mmole) by the Vilsmeier-Haack method according to Jackson and Smith.¹⁶⁶

1-Methyl-3-(N,N-methylimmonio)methyl-1H-indole chloride (4.12).—

To chilled dimethylformamide (25 ml, 325 mmole) in an ice-salt bath was added phosphoryl chloride (5 ml, 54.6 mmole) and the solution was stirred for 10 minutes. N-methylindole (5.25 g, 40 mmole) was added dropwise, the slurry that formed was left stirring for 30 minutes, then carbontetrachloride (50 ml) was added and the mixture was stirred for a further 5 minutes. The white precipitate that formed was filtered off, washed well with carbontetrachloride and dried under a vacuum of 0.1 mm Hg to remove residual dimethylformamide, to give the immonium chloride salt (4.11) (8.78 g, 98.5%). The salt (4.11) was recrystallised by dissolving in a minimum of cold water and pouring this solution into a much larger volume of acetone, to slowly give white needles which did

not melt but at 200°C began to both decompose and sublime, and had $\nu_{\max}$ 1650, 1542, 1439, 1409, 1265, 1136, 1120, 858 and 749 cm^{-1} , PMR (D_2O , internal standard: sodium 3-(trimethylsilyl)-propyl sulphonate) 8.06 (1H, s, H- α or H-2), 7.77 (1H, s, H-2 or H- α), 7.72 (4H, narrow m, H-4, 5, 6, 7), 3.68 (6H, s, C=NMe₂), 3.24 (3H, s, NMe). (Found C, 64.6; H, 6.8; N, 12.5; $\text{C}_{12}\text{H}_{15}\text{ClN}_2$ requires C, 64.7; H, 6.8; N, 12.6%).

1-Methyl-3-(N,N-methylimonio)methyl-1H-indole iodide (4.14).-

To the chloride salt (4.12) (668 mg, 3 mmole) in hot methanol (20 ml) was added sodium iodide (1 g, 6.7 mmole) in methanol (20 ml) and the precipitate which formed was filtered off after cooling the mixture, and dried under vacuum to give the iodide salt (880 mg, 93%).

Recrystallised from methanol, it slowly decomposed at ca. 300°C without melting, and had $\nu_{\max}$ 3090, 1645, 1535, 1406, 1395, 1430, 1260, 1170, 1160, 1140, 1113, 860 and 757 cm^{-1} . (Found, C, 46.0; H, 4.7; I, 40.8; N, 9.2; $\text{C}_{12}\text{H}_{15}\text{IN}_2$ requires C, 45.9; H, 4.8; I, 40.4; N, 8.9%).

The iodide salt (4.14) was also produced when the chloride salt (4.11) was heated for several hours under reflux in methanol with methyl iodide, to give the iodide salt as purple crystals. The purple colour was retained on recrystallisation but the product was shown to be identical with the iodide salt (4.13) from the above preparation by elemental analysis and by comparison of infrared spectra. Heating the purple crystals for at least 12 hours in methanol under reflux gave a white precipitate on cooling that was identical (IR) with the iodide salt (4.13).

Attempted condensation of the immonium salt (4.12) and diethyl N-acetamidomalonate.- The salt (4.12) (669 mg, 3 mmole) and the acetamidomalonate (4.3) (673 mg, 3.1 mmole) were mixed dry, a solution of sodium ethoxide (3.20 mmole) in ethanol (20 ml) was added and the

mixture heated under reflux for 1 hour with the exclusion of atmospheric moisture. The ethanol was evaporated under vacuum to give an orange solid which on analysis by PMR was found to be comprised of the acetamidomalonate (4.3) and 3-formyl-N-methylindole (4.9), the resonances being somewhat broadened by the presence of sodium chloride crystals. The same products were also observed on dilution of the reaction mixture with water after the reflux had ceased, followed by chloroform extraction, nor did heating the mixture for periods up to 12 hours have any effect on the result.

Methyl 2-carbomethoxy-2-(N-methyl-1H-indol-3-yl)acrylate (4.18).-

The immonium salt (4.12) (1.11 g, 5 mmole) and dimethylmalonate (0.73 g, 5.5 mmole) were heated under reflux for 1 hour in dry methanol (20 ml) containing sodium methoxide (0.05 mmole). The methanol was evaporated under vacuum to give a yellow solid that was recrystallised from methanol to give the acrylate (4.18) (0.71 g, 52%). Recrystallised again from methanol, the light yellow needles had m.p. 99-100°C, $\nu_{\max}$ 3130, 1733, 1686, 1601, 1525, 1438, 1328, 1262, 1210, 1076, 1065, 981 and 741 cm^{-1} , PMR (CDCl_3 , *), 8.04 (1H, s, H-2 or H- α), 7.66 (1H, m, H-4), 7.60 (1H, s, H- α or H-2), 7.25-7.10 (3H, m, H-5, 6, 7), 3.85 (3H, s, COOMe), 3.79 (3H, s, COOMe). (Found C, 65.7; H, 5.7; N, 5.0; $\text{C}_{15}\text{H}_{15}\text{NO}_4$ requires C, 65.9; H, 5.5; N, 5.1%).

Attempted condensation of 3-formyl-N-methylindole with dimethylmalonate.- The formylindole (4.9) (1.59 g, 10 mmole) and dimethylmalonate (1.37 g, 10.4 mmole) were dissolved in dry methanol (40 ml) containing sodium methoxide (0.1 mmole) and heated under reflux for one hour. Analysis by t.l.c. (silica gel, CHCl_3 , 1% H_2SO_4 in ethanol spray reagent) showed only the formyl indole (R_f 0.30, purple on

spraying and heating), and no methyl indolacrylate (4.18) (R_f 0.45, orange) was visible. The methanol was evaporated under vacuum, ether was added and the mixture chilled to -20°C . The white precipitate was filtered off and was found by comparison of infrared spectra to be the starting material, 3-formyl-N-methylindole (1.20 g, 75% recovery).

Ethyl 2-carbethoxy-3-(N-methyl-1H-indol-3-yl)acrylate (4.20).-

Sodium (0.29 g, 12.5 mmole) was dissolved in dry ethyl alcohol (50 ml) containing diethyl malonate (1.76 g, 11 mmole) and the immonium salt (4.12) was added. The mixture was stirred at room temperature for 2 hours, diluted with water (150 ml) and extracted three times with chloroform (20 ml), the combined chloroform extracts were dried with sodium sulphate, and vacuum evaporated to give a brown oil. The oil was dissolved in methanol (5 ml) and chilled to -20°C to give the yellow prisms of the malonate (4.20) (1.225 g, 42%). Preparative t.l.c. separation (silica gel, CHCl_3) of the compounds that did not crystallise gave a small amount of the malonate (less than 10 mg) (R_f 0.6) and the remainder comprised the formyl derivative (4.8) (R_f 0.3) and unreacted diethyl malonate (R_f >0.9). Recrystallised from methanol, the yellow prisms had m.p. $75-77^\circ\text{C}$, U_{max} 3110, 1710, 1601, 1534, 1331, 1249, 1216, 1203, 1078, 1014, 948, 849 and 741 cm^{-1} , PMR (CDCl_3 , *), 8.02 (1H, s, H-2 or H- α), 7.66 (1H, m, H-4), 7.60 (1H, s, H- α or H-2), 7.25-7.10 (3H, m, H-5, 6, 7), 4.33 (2H, q, $J=7\text{ Hz}$, CH_2), 4.25 (2H, q, $J=7\text{ Hz}$, CH_2), 3.66 (3H, s, NMe), 1.31 (6H, t, CH_3). (Found, C, 67.9; H, 6.4; N, 4.5; $\text{C}_{17}\text{H}_{19}\text{NO}_4$ requires C, 67.8; H, 6.4; N, 4.7%).

Attempted condensation of diethyl-N-acetamidomalonate with

3-formyl-N-methylindole.- a) The formylindole (4.9) (318 mg, 2 mmole) and the amidomalonate (4.3) (440 mg, 2.03 mmole) were dissolved in dry ethanol (50 ml) containing sodium ethoxide (2.25 mmole) and left stand

for 24 hours. Only the formylindole (4.8) (R_f 0.3) was observed on analysis by t.l.c. (silica gel, CHCl_3). Refluxing this solution for 1 hour did not alter its composition.

b).- The formylindole (4.9) (478 mg, 3 mmole) and the malonate (4.3) (660 mg, 3.04 mmole) in tetrahydrofuran (THF) (20 ml) were added to a mixture of titanium tetrachloride (5.7 mmole) in carbontetrachloride (1.5 ml) and THF (20 ml) then pyridine (250 mg, 6 mmole) was added dropwise over 30 minutes. The mixture was stirred for 15 days, then centrifuged and the supernatant liquid was evaporated under vacuum to give a brown oil which on PMR analysis was found to be largely the malonate (4.18) with some of the formylindole (4.9) also present. The purple precipitate was dissolved in a mixture of water (20 ml) and chloroform (10 ml) by shaking for 10 minutes, then the mixture was extracted twice with chloroform (20 ml) and the combined chloroform extracts evaporated under vacuum to give a brown oil containing some solid, which on PMR analysis was found to be largely the formylindole (4.9) with some of the malonate (4.3) present.

Methyl N-acetyl- β -[4-nitrophenyl]serine (4.24).- p-Nitrobenzaldehyde (454 mg, 3 mmole), monomethyl N-acetylmalonate (539 mg, 3.08 mmole) and triethylamine (303 mg, 3 mmole) were dissolved and left for 12 hours at 50°C. The solvent was then evaporated under vacuum to give an oil which was dissolved in methanol (3 ml) and chilled to -20°C. The fine white fluffy needles obtained were filtered off and dried under vacuum to give the serine (4.24) (507 mg, 60%), with m.p. 182-183°C {lit.,¹⁷³ 176.5-178°C}, ν_{max} 3350, 1754, 1674, 1655, 1540, 1450, 1223, 1147, 1079, 1007, 868 and 721 cm^{-1} , PMR ($d^6\text{DMSO}, *$), 8.05 (2H,d,J=8.6 Hz, H-3 and H-5), 7.52 (2H,d,J=8.6 Hz, H-2 and H-6), 6.1 (1H,bd,J=4 Hz,NH), 5.0-4.3 (2H,m,O-CH-CH-N), 3.56 (3H,s,COOMe), 1.75 (3H,s,NCOMe).

Attempted condensation of the formylindole (4.9) with monoethyl N-acetamidomalonate (4.28).— The formylindole (4.9) (159 mg, 1 mmole), the monoester (4.28) (195 mg, 1.03 mmole) and triethylamine (1.41 mg, 1.4 mmole) were mixed and left for 6 weeks, after which analysis by PMR showed that little, if any, reaction had taken place. The mixture was then heated at 100°C for 30 minutes, but the only effect observed was a much lowered triethylamine content.

Attempted condensation of the immonium salt (4.12) with the monoesters of N-acetylamidomalic acid.— The salt (4.12) (668 mg, 3 mmole), the monoethyl malonate (4.28) (569 mg, 3.01 mmole) and triethylamine (300 mg, 3 mmole) in methanol (10 ml) were left standing for 24 hours at room temperature when analysis by t.l.c. (silica gel, CHCl_3) showed only the salt ($R_f < 0.01$) and the formylindole ($R_f 0.3$) were present. Heating the solution under reflux for 1 hour gave a solution with a chromatogram identical with the first chromatogram.

A similar attempt using the monomethyl ester (4.23) was also unsuccessful.

Attempted reduction of the chloride salt (4.12).—

a) Sodium Borohydride.— The salt (4.12) (1.11 g, 5 mmole) was added to methanol (25 ml) and cooled to -20°C, then sodium borohydride (95 mg, 2.5 mmole) was added and the mixture stirred for 30 minutes as it warmed to room temperature. The salt (4.12) largely dissolved and the residual solid was filtered off and the filtrate evaporated under vacuum to give a solid which was found to be the immonium salt starting material by comparison of infrared spectra.

b) Sodium dissolving in methanol.— Sodium metal (0.6 g, 26 mmole) was added in small lumps over 5 minutes to the immonium salt (4.12) (0.891 g, 4 mmole) in methanol (10 ml). During the addition there was

vigorous gas evolution and the flask became hot, and, after the addition was complete, the solution was cooled, diluted with water, then extracted twice with chloroform (10 ml). The combined chloroform extracts were dried with sodium sulphate and evaporated under vacuum to give a solid which proved to be 3-formyl-N-methylindole by analytical t.l.c. and infrared spectral evidence.

c) Lithium aluminium hydride.- Tetrahydrofuran (THF) was dried by heating under reflux for 30 minutes over lithium aluminium hydride (LAH), then the solvent (10 ml) was distilled into a flask containing the immonium salt (4.12) (1.56 g, 7 mmole) and LAH (0.4 g, 10 mmole). The flask was left stirring for 60 hr at room temperature, then water (0.5 ml) and 10% sodium hydroxide (2 ml) were added, the precipitated solid was filtered off, and the filtrate was diluted with water (100 ml). The aqueous mixture was extracted twice with chloroform (10 ml), dried with sodium sulphate, and evaporated under vacuum to give an oil (1.352 g). On analysis by t.l.c., the oil was found to be a complex mixture with two major components, one of which was 3-formyl-N-methylindole (4.9) and the other, which was isolated by preparative t.l.c., was found to be 1-methylgramine (4.37) (0.15 g, 9%), PMR (CCl_4 , *), 7.55 (1H, m, H-4), 7.1-6.9 (3H, m, H-5, 6, 7), 6.65 (1H, s, H-2), 3.44 (2H, s, $\text{CH}_2\text{-N}$), 3.44 (3H, s, NMe), 2.14 (6H, s, NMe_2).

When the immonium salt (4.12) was heated with LAH under reflux in THF for 12 hours, again a complex mixture was obtained of which the formyl derivative (4.9) was again the largest component.

Part 5 Immunological Studies using the Synthesised Haptens

Discussion

The two haptens (2.1) and 3.20) synthesised in this present work, were coupled to carriers for use as antigens. The aminoalcohol (2.1) was found to be most easily

handled as the boronate (2.23) (page 52)

and coupling was carried out by

hydrolysing the boronate to the

free amine (2.1) and then diazotising

and coupling by a standard procedure.¹⁷⁹

Diazotised species couple mainly to

lysine, histidine and tyrosine¹⁷⁹

(page 32), and the determinant groups on an antigen resulting from

coupling 2.1 to a protein will therefore be mainly the groups such

as 5.1 and 5.2. The acid (3.20) was coupled by use of dicyclohexyl-

carbodiimide (DCC) (page 33), with amino and alcohol groups on the

protein, to give in the case of lysine, the determinant group (5.3).

Apart from the initial experiments in coupling the aminoalcohol (2.1) to bovine γ -globulin, which were carried out by this author, all coupling of haptens and immunological testing was carried out by a group lead by Dr W. E. Jonas.⁸⁹ To date, there have been three sets of tests, two on the haptens above (2.1 and 3.20) and a set of tests using a hapten derived from sporidesmin-A (page 38). Guinea-pigs, rabbits and sheep have been used as test subjects in each of the three immunisation programs, but the results are mainly from tests carried out on pooled guinea-pig sera, since the workers have found that a good response by sheep to an antigen is generally only obtained when guinea-pigs have also been found sensitive to the same antigen. A difficulty in the work has been the requirement that a high average response be obtained. Often when such work has been carried out in the past, the prime interest has been in the nature of the antibodies produced, and results are quoted for the few high yielding animals in a group. In the present project, the aim is to obtain a vaccine for use in immunising the majority of animals, and so a high average response is required.

The sporidesmin-A derived hapten which was tested by the indirect haemagglutination test (page 36), demonstrated that antibodies could be produced, but that a practical immunogenic level had not been achieved.⁸⁹ Similar results were obtained for a program using the aminoalcohol (2.1), which was evaluated using the complement fixation test (page 36), and the antigen derived from the acid (3.20) failed to induce a detectable response. These tests were all carried out on sera resulting from immunisation programs using proteins as hapten carriers. Recently it has been found that mycobacteria are far better carriers than proteins and quite good responses have been achieved by this modification. Of a

variety of bacteria tested for their effectiveness, the best response has been found with *Mycobacterium phlei*.¹⁸⁰ These tests have all used the aminoalcohol (2.1) as a standard hapten, and the cross-haptens used to test specificity are drawn from a number of sources, in particular from the degradation of sporidesmin-A.

The results, given in table 5.1, show that the concentration of antibody specific to the determining (aminoalcohol) group is much greater for that coupled to the mycobacteria than for that coupled to the small bovine gamma-globulin (BYG) protein. (Line labelled "none"). The figures represent the degree of dilution required before a minimum detectable concentration of antibody is reached. Thus a titre of 80 means that the guinea-pig serum containing the antibodies must be diluted to 1/80th of its original concentration to reach the minimum detectable activity level. The far more active anti-mycobacterium-aminoalcohol antibody requires a 2560-fold dilution to reach the minimum level. The word "titre" will thus be seen to be used here in a rather different sense from that used in analytical chemistry. It should also be realised that the scale is more properly regarded as logarithmic, and each value is only approximate, the apparent value 2560 meaning a titre between 1800 and 3600, and a value of 80 meaning a titre between 57 and 113. (See footnote).

These results imply that a method has been developed for generating a practical level of antibody response. The next objective, to obtain a set of antibodies reactive with sporidesmin-A, has yet to

Footnote: The figures in table 5.1 except the value 5 all belong to the series $2^n \times 10$ where n is an integer, and this is a result of diluting and rediluting the sera by 2, after an initial 10-fold dilution, so that a figure $2^n \times 10$ expresses a titre between $2^{n-\frac{1}{2}} \times 10$ and $2^{n+\frac{1}{2}} \times 10$.

Table 5.1

Hapten-inhibition complement fixation titre

Inhibiting cross-hapten	Complement fixation titre (Reciprocal) using guinea-pig anti-	
	B G-alcohol (2.1)	<i>M.phlei</i> -alcohol (2.1)
a None	80	2560
b aminoalcohol (2.1)	5	5
c phenylboronate (2.23)	20	20
d isatin 5.4	10	80
e 5-chlorovanillin (2.4)	20	160
f 5-chloroindole acid (3.19)	20	160
g sporidesmin-A (1.1)	20	320
h indigo (5.5)	40	320
i tryptophan (5.6)	80	640
j dioxopiperazine (5.7)	80	1280

2.1

2.23

5.4

2.4

3.19

5.6

1.1

5.5

5.7

be achieved, as can be seen from the results of inhibiting the antibody response by use of cross-haptens. The addition of a cross-hapten compound to an antibody-containing serum may cause the antibody binding site to be blocked, and effectively inhibits the antibody from reacting with the test antigen (in this case, aminoalcohol coupled to keyhole limpet haemocyanin). Pre-addition of the aminoalcohol (2.1) to the sera resulted in a strong binding reaction, since only 5-fold dilution was required to obtain the (unblocked) minimum detectable antibody concentration. On the other hand, pre-addition of sporidesmin-A resulted in rather weak binding, and neither the protein nor the *M. phlei* antisera could be regarded as showing strong sporidesmin-A interactions.

The next step should be to couple sporidesmin-A to *M. phlei*, which would hopefully give a sporidesmin-specific antigen, and demonstrate that a practical vaccine can be obtained. Since sporidesmin-A is difficult to obtain in quantity, this would then offer a challenge to the synthetic organic chemist to devise a means of synthesising a sporidesmin-like hapten in kilogram quantities.

Experimental

The majority of the work in this part was done by a group under Dr W. E. Jonas, Wallaceville Animal Research Centre, New Zealand, and the experimental details will be reported elsewhere.¹⁸⁰ Coupling of the phenylboronate, however, was carried out by the author of this present work, in the following manner.

The phenylboronate (2.23) (40 mg, 0.13 mmole) was dissolved in warm methanol, concentrated hydrochloric acid (1 drop) was added, the solution was left to stand for 5 minutes, then cooled to 4°C and a further 5 drops of concentrated hydrochloric acid were added. Sodium nitrite, 0.75 ml of a standard solution of 14 mg/ml, 0.15 mmole) was added and the solution was again left to stand for 5 minutes at 4°C. The diazonium solution was then added dropwise to a solution of bovine gamma-globulin (1 g) (Armour Pharmaceutical Company) in a buffer solution (20 ml) consisting of 0.13 M sodium chloride, 0.16 M boric acid, and sufficient sodium hydroxide to raise the pH to 9. After each addition of a few drops, the pH (which was constantly monitored by a meter) was readjusted to between 9.0 and 9.5 by the addition of 0.5 M sodium hydroxide. After the addition was complete, the solution was left stirring at 4°C for an hour with further additions of sodium hydroxide as necessary to maintain the pH in the range 9.0 to 9.5. The protein-hapten solution was then eluted through a column of Sephadex G-25 by the buffer solution, and dialysed 5 times in the cold against borate buffer (1 L, pH 9) for a total of 48 hours. The ultraviolet spectrum of the protein was measured (for a concentration sufficient to give an optical density of 1 at 280 nm) and compared with the spectrum of similarly normalised bovine gamma-globulin. The pale orange protein-hapten solution had in addition to the bands observed for the non-coupled

protein, a broad band from 320 to 360 nm, with a maximum optical density of 0.3.

The immunisation program carried out by Dr Jonas and his coworkers used three different concentrations of protein-hapten solution (for guinea-pigs; 3.6, 0.72 and 0.14 mg per injection) mixed with Freund's complete adjuvant (page 32). The immunisation schedule was: day 1, 0.2 ml into each hind foot pad; days 10 and 20, 0.2 ml intramuscularly into each hind leg; day 30, bled out. All antisera were heated at 56°C for 30 minutes to destroy the complement present, then stored at -20°C.

REFERENCES

1. J. G. Gilruth, New Zealand Dept of Agric. Annual Report, 1908, 16, 189.
2. J. F. Filmer, N.Z. J. Agric., 1958, 97, 207.

The New Zealand Journal of Agriculture is produced for farmers, and much of the history of facial eczema is outlined in it.

The New Zealand Journal of Agricultural Research contains scientific papers.
3. R.L.M. Syngé and E. P. White, Chem. Ind. (London), 1959, 1546.
4. J. W. Ronaldson, A. Taylor, E. P. White and R. J. Abraham, J. Chem. Soc., 1963, 3172.
5. J. Fridrichsons and A. McL. Mathieson, Tetrahedron Lett. 1962, 1265.
6. J. Fridrichsons and A. McL. Mathieson, Acta Crystallogr., 1965, 18, 104.
7. A. F. Beecham, J. Fridrichsons and A. McL. Mathieson, Tetrahedron Lett., 1966, 3131.
8. N. R. Towers, Proc. Symp. N.Z. Inst. Chem., (Manawatu), August, 1975.
9. C. H. Gallagher, Biochem. Pharmacol., 1964, 13, 1017.
10. J. L. Richard, J. Amer. Vet. Med. Assoc., 1973, 163, 1298.
11. D. P. Sinclair and M. W. Howe, N.Z. J. Agr. Res., 1968, 11, 59.
12. V. J. Grenda, R. E. Jones, G. Gal and M. Sletzing, J. Org. Chem., 1965, 30, 259.
13. B. S. Janes, Nature (London), 1959, 184, 1327.
14. A. Taylor, "Microbial Toxins", Vol. VII, S. Kadis, A. Ciegler and S. J. Ajl, eds., Academic Press, New York, 1971, 337.
15. W. A. Te Punga, N.Z. J. Agr., 1962, 105, 39.
16. A. Taylor, "Biochemistry of Some Foodborne Microbial Toxins", R. I. Mateles and G. N. Wogan, eds., M.I.T. Press, Cambridge, Mass., 1967, 69.

17. P. G. Sammes, Fortschritte der Chemie Organischer Naturstoffe, 1975, 32, 51.
18. R. Hodges, J. W. Ronaldson, A. Taylor and E. P. White, J. Chem. Soc., 1963, 5332.
19. R. Hodges, J. W. Ronaldson, J. S. Shannon, A. Taylor and E. P. White, J. Chem. Soc., 1964, 26.
20. R. Hodges and A. Taylor, J. Chem. Soc., 1964, 4310.
21. H. Herrman, R. Hodges and A. Taylor, J. Chem. Soc., 1964, 4315.
22. G. Lowe, A. Taylor and L. C. Vining, J. Chem. Soc. (c), 1966, 1799.
23. R. Hodges, J. S. Shannon and A. Taylor, J. Chem. Soc. (c), 1966, 1803.
24. W. D. Jamieson, R. Rahmen and A. Taylor, J. Chem. Soc. (c), 1969, 1564.
25. R. Rahmen, S. Safe and A. Taylor, J. Chem. Soc. (c), 1969, 1664.
26. S. Safe and A. Taylor, J. Chem. Soc. (c), 1970, 432.
27. S. Safe and A. Taylor, J. Chem. Soc. (c), 1971, 1189.
28. E. Francis, R. Rahmen, S. Safe and A. Taylor, J. Chem. Soc., Perkin Trans. I, 1972, 470.
29. S. Safe and A. Taylor, J. Chem. Soc., Perkin Trans. I, 1972, 472.
30. J. W. Ronaldson, Aust. J. Chem., 1975, 28, 2043.
31. J. W. Ronaldson, Aust. J. Chem., 1976, 29, 2307.
32. R. Hodges and J. S. Shannon, Aust. J. Chem., 1966, 19, 1059.
33. M. Przybylska and E. M. Gopalakrishna, Acta Crystallogr. Sect. B., 1974, 30, 597.
34. M. R. Bell, J. R. Johnson, B. S. Wildi and R. B. Woodward, J. Am. Chem. Soc., 1958, 80, 1001.
35. G. M. Strunz, M. Kakushima and M. Stillwell, Can. J. Chem., 1975, 53, 295.
36. N. Neuss, R. Nagarajan, B. B. Molloy and L. L. Huckstep, Tetrahedron Lett., 1968, 4467.

37. N. R. Towers and D. E. Wright, N.Z. J. Agric. Research, 1969, 12, 275.
38. G. W. Kirby and M. J. Varley, J. Chem. Soc., Chem. Commun., 1974, 833.
39. P. J. Brook and R.E.F. Mathews, N.Z. J. Sci., 1960, 3, 591.
40. P. W. Trown, Biochem. Biophys. Res. Commun., 1968, 33, 402.
41. I. D. Rae and B. N. Umbrasas, Aust. J. Chem., 1971, 24, 2729.
42. P. M. Pojer and I. D. Rae, Aust. J. Chem., 1972, 25, 1737.
43. R. G. Amiet, F. W. Eastwood and I. D. Rae, Aust. J. Chem., 1972, 25, 1473.
44. H.C.J. Ottenheym, T. F. Spande and B. Witkop, J. Am. Chem. Soc., 1973, 95, 1989.
45. P. Rosenmund and K. Kaiser, Angew. Chem. Int. Ed. Engl., 1970, 9, 162.
46. G. P. Slater, Chem. Ind. (London), 1969, 1092.
47. O. Grahl-Neilson, Tetrahedron Lett., 1969, 2827.
48. T. Hino and T. Sato, Chem. Pharm. Bull., 1974, 22, 2866.
49. T. Sato and T. Hino, Chem. Pharm. Bull., 1976, 24, 285.
50. T. Sato and T. Hino, Tetrahedron, 1976, 32, 507.
51. H. Poisel and U. Schmidt, Angew. Chem. Int. Ed. Engl., 1971, 10, 130.
52. H. Poisel and U. Schmidt, Chem. Ber., 1971, 104, 1714.
53. H. Poisel and U. Schmidt, Chem. Ber., 1972, 105, 625.
54. E. Ohler, H. Poisel, F. Tataruch and U. Schmidt, Chem. Ber., 1972, 105, 635.
55. E. Ohler, F. Tataruch and U. Schmidt, Chem. Ber., 1972, 105, 3658.
56. E. Ohler, F. Tataruch and U. Schmidt, Chem. Ber., 1973, 106, 165.
57. E. Ohler, F. Tataruch and U. Schmidt, Chem. Ber., 1973, 106, 396.
58. H. Poisel and U. Schmidt, Chem. Ber., 1973, 106, 3408.
59. J. Hausler and U. Schmidt, Chem. Ber., 1974, 107, 145.
60. D. Scholz and U. Schmidt, Chem. Ber., 1974, 107, 2295.
61. J. Hausler and U. Schmidt, Chem. Ber., 1974, 107, 2804.
62. U. Schmidt, A. Perco and E. Ohler, Chem. Ber., 1974, 107, 2816.

63. U. Schmidt and A. Nikiforov, Monatsh. Chem., 1975, 106, 313.
64. H. Poisel and U. Schmidt, Chem. Ber., 1975, 108, 2547.
65. E. Ohler and U. Schmidt, Chem. Ber., 1975, 108, 2907.
66. H. Poisel and U. Schmidt, Chem. Ber., 1975, 108, 2917.
67. I. Ugi, Angew. Chem. Int. Ed. Engl., 1962, 1, 9.
68. Y. Kishi, T. Fukuyama and S. Nakatsuka, J. Am. Chem. Soc., 1973, 95, 6490.
69. Y. Kishi, T. Fukuyama and S. Nakatsuka, J. Am. Chem. Soc., 1973, 95, 6492.
70. Y. Kishi, T. Fukuyama, S. Nakatsuka and M. Havel, J. Am. Chem. Soc., 1973, 95, 6493.
71. S. Nakatsuka, T. Fukuyama and Y. Kishi, Tetrahedron Lett., 1974, 16, 1549.
72. Y. Kishi, M. Aratani, H. Tanino, T. Fukuyama, T. Goto, S. Inoue, S. Sugiura and H. Kakoi, J. Chem. Soc., Chem. Commun., 1972, 64.
73. Y. Kishi, private communication.
74. I. M. Roitt, "Essential Immunology" (2nd edn), Blackwell Scientific Publications, 1974,
75. P. L. Carpenter, "Immunity and Serology" (3rd edn), W. B. Saunders Cpy, Philadelphia, 1975; a) p 16, b) p 135, c) p 310, d) p 160, e) p 186, f) p 232 and p 324.
76. L. Weiss, "The Cells and Tissues of the Immune System, Structure, Function, Interaction", Prentice-Hall, New Jersey (1972).
77. E. D. Day, "Foundations of Immunochemistry", Williams and Wilkins, Baltimore, 1966, a) p 32, b) p 14.
78. M. W. Steward, "Immunochemistry", Chapman and Hall, London, 1974; a) p 26, b) p 25, c) p 30, d) p 28, e) p 9, f) p 16.
79. R. R. Porter, Scientific American, Oct. 1967, 81.
80. G. M. Edelman, Scientific American, Aug. 1970, 34.

81. N. K. Jerne, Scientific American, Jul. 1973, 53.
82. The Nobel Foundation and W. Odelberg, eds, "Nobel, The Man and his Prizes", (3rd edn), Elsevier, New York, 1972, p166.
83. K. Landsteiner, "The Specificity of Serological Reactions", Harvard Univ. Press, Cambridge, Mass., 1945.
84. D. Pressman and A. L. Grossberg, "The Structural Basis of Antibody Specificity", Benjamin, New York, 1968, a) p 30, b) p 6, c) p 19 and p 242.
85. P. Jolles and A. Paraf, "Chemical and Biological Basis of Adjuvants", Springer-Verlag, New York, 1973.
86. J. Freund, Fortschritte, Tuberk.-Forsch., 1956, 7, 130.
87. W. E. Jonas, personal communication. (Detailed procedures of most coupling reactions will be found in reference 179.)
88. M. M. Mayer, Scientific American, Nov. 1973, 54.
89. W. E. Jonas and J. W. Ronaldson, N.Z. J. Vet. Sci.,
90. A. Standen, H. F. Mark, J. J. McKetta and D. F. Othmer, eds., "Kirk-Othmer Encyclopedia of Chemical Technology", 2nd edn, 1970, Wiley and Sons, New York.
91. S. Yokoyama, T. Ishii, G. Takeya, A. Sakakibara, Kogyo Kagaku Zasshi, 1969, 346.
92. H. D. Ludeman and H. Nimz, Biochem. Biophys. Res. Commun., 1973, 52, 1162.
93. G. Aulin-Erdtman, Sv. Papperstidn., 1953, 56, 91. (CA 1955, 49, 8209e).
94. W. C. McCrone, Anal. Chem., 1950, 22, 500.
95. H. O. Mottern, J. Am. Chem. Soc., 1934, 56, 2017.
96. L. C. Raiford and J. G. Lichty, J. Am. Chem. Soc., 1930, 52, 4576.
97. L. F. Feiser and M. Feiser, "Reagents for Organic Synthesis", 1967, Wiley and Sons, New York. a) 90, b) 1079, c) 1049, d) 833.
98. S. R. Hartshorn and K. Schofield, Progr. Org. Chem., 1973, 8, 278.
99. I. A. Pearl, Org. Syn., 1950, 30, 101.

100. L. C. Raiford and D. J. Potter, J. Am. Chem. Soc., 1933, 55, 1682.
101. I. A. Pearl, J. Org. Chem., 1947, 12, 85.
102. I. A. Pearl, J. Am. Chem. Soc., 1946, 68, 429.
103. X. A. Dominguez, G. L. Diaz, F. V. Gonzalez, Ciencia (Mexico) 1955, 15, 208.
104. S. G. Sunderwirth, private communication to L. F. Feiser and M. Feiser.⁹⁷
105. L. C. Raiford and R. C. Perry, J. Org. Chem., 1942, 7, 354.
106. J. S. Buck, Org. Syn., 1933, 13, 102.
107. R. J. Ferrier, J. Chem. Soc., 1961, 2325.
108. R. H. Cragg and A. F. Weston, J. Chem. Soc., Dalton Trans., 1975, 2, 93.
109. R. H. Cragg and M. Nazery, J. Chem. Soc., Dalton Trans., 1974, 1, 162.
110. F. G. Mann and B. C. Saunders, "Practical Organic Chemistry", Longmans, London, 4th edn, 1960. a) p 180, b) p 241.
111. L. C. Raiford and D. E. Floyd, J. Org. Chem., 1943, 8, 358.
112. K.I.H. Williams, S. E. Cremer, F. W. Kent, E. J. Sehm and D. S. Tarbell, J. Am. Chem. Soc., 1960, 82, 3982.
113. S. C. Cummings and D. H. Busch, Inorg. Chem., 1971, 10, 1220.
114. L. I. Smith and J. W. Opie, Org. Syn., 1948, 28, 11.
115. R. L. Augustine, "Catalytic Hydrogenation", Marcel Dekker, New York, 1965. a) p 128, b) p 83.
116. I. I. Bat, G. A. Chistyakova, P. N. Ovchinnikov, V. V. Rebrova, L. G. Guseva and S. S. Shcherbakova, Tr., Gos. Inst. Prikl. Khim., 1969, 63. (CA 1970; 73, 34930).
117. M. Brink, Acta. Univ. Lund., Sect. II, 1965, paper no. 16.
118. P. Crooij, South African Patent No. 68 03,083, 11 Nov., 1968. (CA 1969; 71/822327).
119. M. Julia, P. Manoury and C. Voillaume, Bull. Soc. Chim. France, 1965, 1417.

120. L. C. Raiford and W. C. Stoesser, J. Am. Chem. Soc., 1928, 50, 2556.
121. L. C. Raiford and M. Ravelly, J. Org. Chem., 1940, 5, 204.
122. E. M. Hindmarsh, I. Knight and R. Robertson, J. Chem. Soc., 1917, 940.
123. D. Cardwell and R. Robertson, J. Chem. Soc., 1915, 255.
124. R.A.B. Bannard and G. Latremouille, Can. J. Chem., 1953, 31, 469.
125. F. R. Graesser-Thomas, J. M. Gulland and R. Robertson, J. Chem. Soc., 1926, 1971.
126. T. Heap, T.G.H. Jones, and R. Robertson, J. Chem. Soc., 1927, 2021.
127. P. Rayet, M. Prost and M. Urbain, Helv. Chim. Acta, 1956, 39, 87.
128. R. Pschorr, Ann., 1912, 391, 23. (J. Chem. Soc., Abstr., 1912, Ai, 775).
129. C. A. Fletscher, Org. Syn., 1953, 33, 65.
130. H. H. Hodgson and H. G. Beard, J. Chem. Soc., 1926, 154.
131. F. B. Whittmer and L. C. Raiford, J. Org. Chem., 1945, 10, 527.
132. R. A. Heacock and O. Hutzinger, J. Chem. Soc., 1963, 3574.
133. H. Gottlieb, personal communication.
134. D. H. Williams and I. Fleming, "Spectroscopic Methods in Organic Chemistry", McGraw-Hill, London, 2nd edn, 1973. a) p 136, b) p 126, c) p 140.
135. L. M. Jackman and S. Sternhell, "Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry", Pergamon Press, London, 2nd edn, 1969. a) p 202, b) p 83.
136. C. H. Ludwig, B. J. Nist and J. L. McCarthy, J. Am. Chem. Soc., 1964, 86, 1186.
137. N. S. Bhacca, L. F. Johnson and J. N. Shoolery, "Varian N.M.R. Spectra Catalogue", National Press, 1962. a) Spectrum no. 197, b) p 8.
138. G. C. Levy and G. L. Nelson, "Carbon-13 Nuclear Magnetic Resonance for Organic Chemists", Wiley-Interscience, New York, 1972. a) p 81.

139. K. S. Dhami and J. B. Stothers, Can. J. Chem., 1966, 44, 2855.
140. D. D. Perrin, W.L.F. Armarego and D. R. Perrin, "Purification of Laboratory Chemicals", Pergamon Press, London, 1966.
141. J. A. McRae, M. Allen and R. Y. Moir, Can. J. Chem., 1961, 39, 995.
142. H. G. Kuivila and A. R. Hendrickson, J. Am. Chem. Soc., 1952, 74, 5068.
143. B. M. Mikhailov and V. A. Vaver, Doklady Akad. Nauk. SSSR., 1955, 102, 531.
144. R. M. Washburn, E. Levens, C. F. Albright, F. A. Billig and E. S. Cernak, Advances in Chem. Ser., 1959, 23, 102.
(CA 1960, 54, 6598i).
145. J. H. Boyer and L. R. Morgan, Jr, J. Org. Chem., 1961, 26, 1654.
146. D. H. Hey and L. C. Lobo, J. Chem. Soc., 1954, 2246.
147. S. F. McDonald, J. Chem. Soc., 1948, 376.
148. R. J. Sundberg, "The Chemistry of Indoles" Academic Press, New York, 1970. a) p 182, b) p 365, c) p 19, d) p 159, e) p 219.
149. R. K. Brown, Chem. Heterocycl. Compounds, 1972, 25, 227.
150. F. Benington, R. D. Morin and L. C. Clark, J. Org. Chem. 1959, 24, 917.
151. A. De Antoni, G. Allegri and C. Costa, Gazz. Chim. Ital., 1970, 100, 1056.
152. I. Baxter and G. A. Swan, J. Chem. Soc. (c), 1968, 468.
153. H. Burton and J. A. Duffield, J. Chem. Soc., 1949, 78.
154. P. J. Black and M. L. Heffernan, Aust. J. Chem., 1965, 18, 353.
155. J.R.A. Pollock and R. Stephens, "Dictionary of Organic Compounds", 1965, Eyre and Spottiswoode. p1845.
156. K. T. Potts and J. E. Saxton, Org. Syn., 1960, 40, 68.
157. W. A. Remers, Chem. Heterocycl. Compounds, 1972, 25, 1.
158. J. M. Gulland, R. Robinson, J. Scott and S. Thornley, J. Chem. Soc., 1929, 2924.

159. A. Buzas, C. Hoffman and G. Regnier, Bull. Soc. Chim. France, 1960, 643.
160. R. L. Hinman and J. Lang, J. Am. Chem. Soc., 1964, 86, 3796.
161. B. Heath-Brown and P. G. Philpott, J. Chem. Soc., 1965, 7185.
162. H. N. Rydon and J. C. Tweedle, J. Chem. Soc., 1955, 3499.
163. K. Chamberlain and R. L. Wain, Ann. Appl. Biol., 1973, 75, 409.
164. A. Da Settimo and E. Nannipieri, J. Org. Chem., 1970, 2546.
165. P. N. James and H. R. Snyder, Org. Syn. Coll. Vol. IV, 1943, 539.
166. A. H. Jackson and A. E. Smith, J. Chem. Soc., 1964, 5510.
167. S. Seshadri, J. Sci. Ind. Res. (India), 1973, 32, 128.
168. G. F. Smith, J. Chem. Soc., 1954, 3842.
169. E. Wenkert, private communication.
170. J. D. Albright and H. R. Snyder, J. Am. Chem. Soc., 1959, 81, 2239.
171. W. Lehnert, Tetrahedron, 1972, 28, 663.
172. W. Lehnert, Tetrahedron, 1973, 29, 635.
173. H. Hellmann and H. Piechota, Ann., 1960, 631, 175.
174. H. R. Snyder and E. L. Eliel, J. Am. Chem. Soc., 1948, 70, 1703.
175. S. Klutchko, H. V. Hansen and R. I. Meltzer, J. Org. Chem., 1965, 30, 3454.
176. H. Hellman and F. Lingens, Z. Physiol. Chem., 1954, 297, 283.
177. H. Hellmann, K. Teichmann and F. Lingens, Chem. Ber., 1958, 91, 2427.
178. J. W. Cornforth and H. T. Huang, J. Chem. Soc., 1948, 1960.
179. A. Nisonoff, "Methods in Immunology and Immunochemistry",
C. A. Williams and M. W. Chase, eds., Vol. I, p.120.
180. W. E. Jonas and A. F. Erasmuson, in preparation.