

STRUCTURE AND BIOSYNTHESIS OF CAPSAICIN

A Thesis submitted by

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ABSTRACT

Capsaicin, the pungent principle of Capsicum annuum, has been shown, by chemical and spectroscopic methods, to be a mixture of closely related compounds. The major, olefinic, component is accompanied by a higher homologue and a double bond isomer. The corresponding dihydro derivative is also present together with lower and higher homologues.

The biosynthesis of the aromatic portion of capsaicin has been investigated using various tritium-labelled precursors. Phenylalanine, various oxygenated cinnamic acids and vanillylamine were found to serve as precursors of the aromatic C_6-C_1 unit. Tyrosine was incorporated much less efficiently than phenylalanine. The possible biogenetic origin of the fatty acid moiety in capsaicin is discussed.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. G.W. Kirby for his guidance and inspiration during this work.

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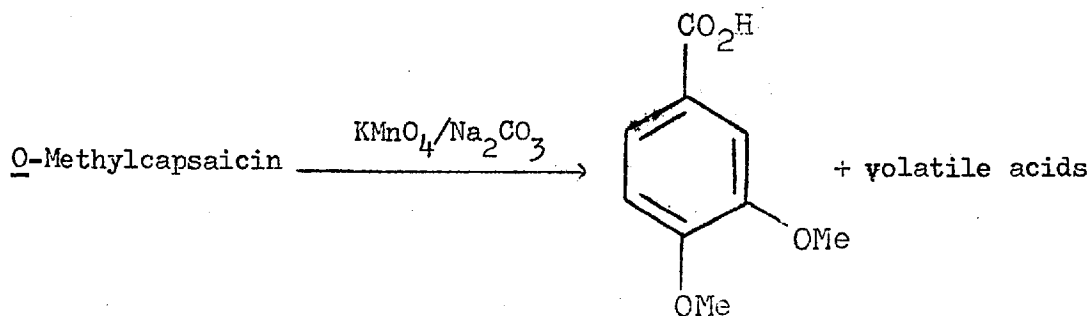
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The Chemistry of Capsaicin

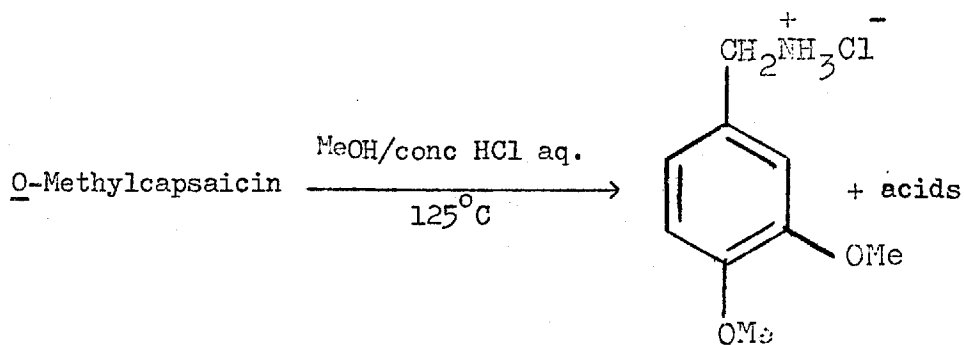
Investigations into the nature of the pungent principle of various peppers were first started at the beginning of the 19th century^(1,2) when an alkali-soluble, red, pungent oil, called 'capsicol', was isolated from cayenne pepper. This oil was later investigated in more detail by Thresh⁽³⁾ who, using the fact that it was soluble in alkali, obtained the pungent principle as a white crystalline solid with a melting point of 62° . This compound, which he called capsaicin, analysed for $C_9H_{14}O_3$, only a carbon and hydrogen analysis being carried out. It was then shown by Micko⁽⁴⁾ that capsaicin contained one methoxyl group and that Thresh had overlooked the presence of nitrogen in capsaicin. Micko obtained the molecular formula $C_{18}H_{28}NO_3$ and a molecular weight of 310 or 643, depending on the strength of the solution. Schotten-Bauman benzylation gave a mono-benzoate, confirming the presence of one phenolic hydroxyl group in the molecule. Treatment with platinic chloride in ethanol gave a residue with a "vanilla odour" indicating the presence of a vanillin residue in the molecule.

The structural work proper was first started by Nelson⁽⁵⁾ who prepared the O-methyl ether derivative and degraded it as follows:-

- 1) Oxidation with alkaline permanganate gave veratric acid and volatile acids,

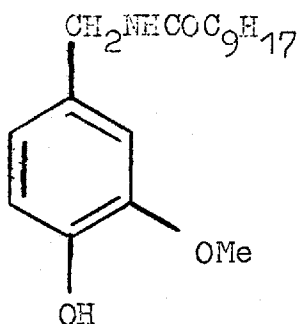


2) Hydrolysis with aqueous methanolic hydrochloric acid gave veratrylamine hydrochloride and unidentified acids.

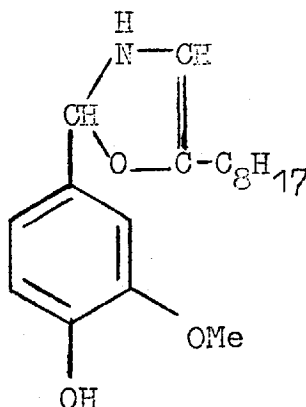


Similarly, hydrolysis of capsaicin with aqueous methanolic hydrochloric acid gave vanillylamine hydrochloride. Alkaline hydrolysis of capsaicin yielded an unsaturated acid, $\text{C}_{10}\text{H}_{18}\text{O}_2$, which

reacted with one molecule of bromine and took up one molecule of hydrogen on catalytic hydrogenation. This reduced acid was not capric acid (mixed melting point). Hence Nelson concluded that capsaicin was a condensation product of vanillylamine and a decenoic acid:-

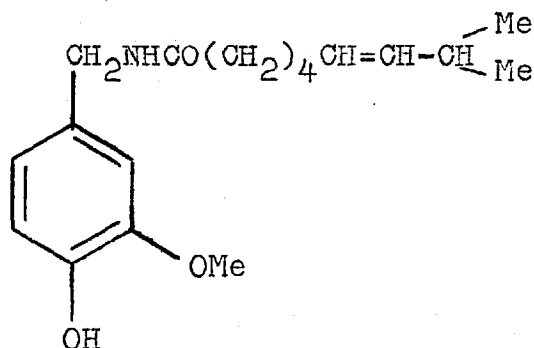


Nelson also pointed out that the formula obtained by Micko, $C_{18}H_{28}NO_3$, was impossible and that the correct formula was $C_{18}H_{27}NO_3$. In addition to this investigation into the structure of capsaicin, Nelson⁽⁶⁾ also synthesised various acylated vanillylamines to test their pungencies. He showed that the pungency was greatest when the side chain was nine or ten carbon atoms long, and that the presence of unsaturation was not necessary for pungency. At the same time, other workers⁽⁷⁾ considered that Nelson's method of degradation was too severe and that some rearrangement had taken place. They suggested that capsaicin was a derivative of a dihydro-oxazole, as follows:-



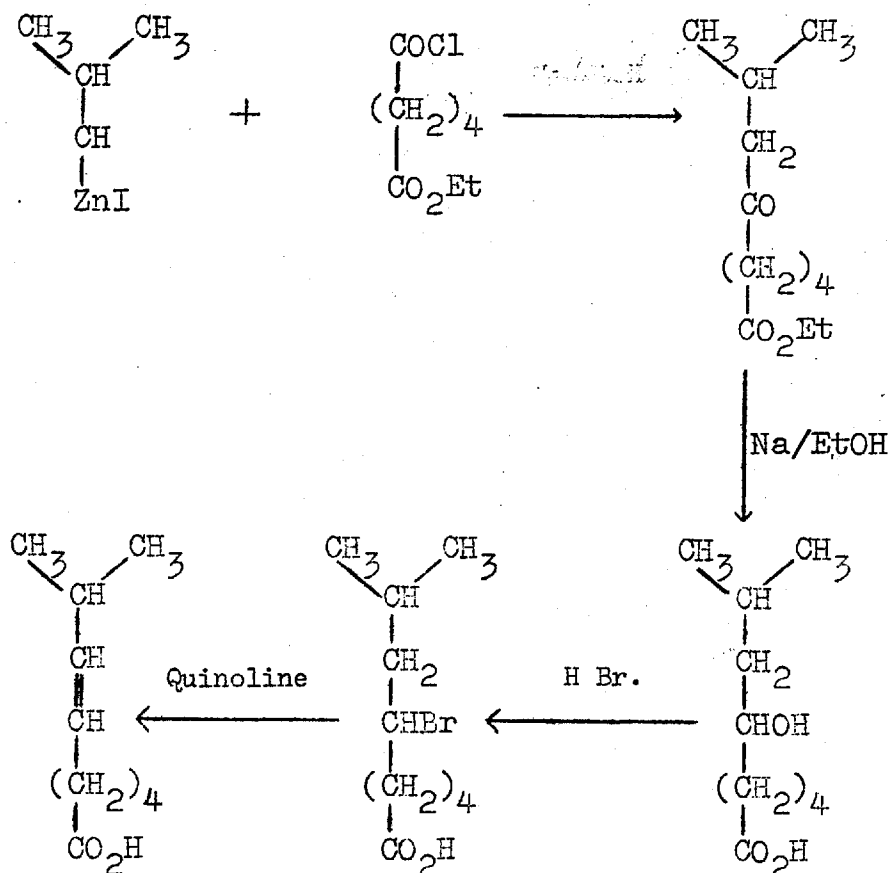
This was disproved by Nelson⁽⁸⁾ who converted the acid, obtained by hydrolysis of capsaicin, into its acid chloride, and condensed it with vanillylamine to give capsaicin, identical to the natural product. Nelson also showed that potash fusion of the acid derived from capsaicin gave acetic acid and a branched chain octanoic acid, but he considered that migration of the double bond would occur under these conditions. He therefore decided to identify the hydrogenated acid by comparing it with known branched chain C_{10} fatty acids. He showed that the acid was 8-methyl-nonanoic acid which, on conversion to its acid chloride and condensation with vanillylamine, gave dihydrocapsaicin, identical to that obtained by hydrogenation of natural capsaicin. The position of the double bond was finally established, by oxidation of the unsaturated fatty acid, which gave adipic acid and isobutyric acid.⁽⁹⁾ Therefore capsaicin is

N-Vanillyl-8-methyl-6-nonenamide:-



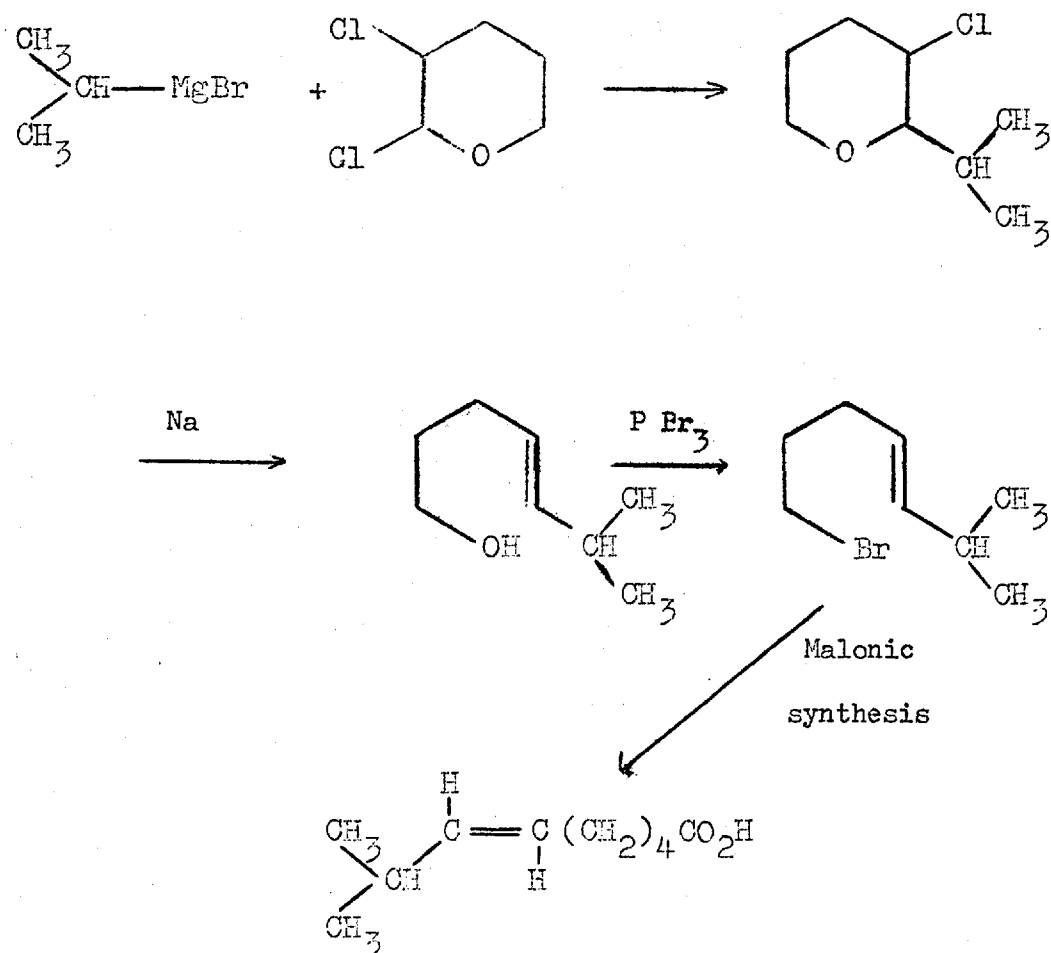
Subsequent work by these investigators was merely concerned with the synthesis of analogues of capsaicin to find the structural features necessary for pungency.

With the structure of capsaicin established, the next step was clearly a synthesis of capsaicin by an unambiguous route which would confirm its structure. The first synthesis was due to Späth and Darling,⁽¹⁰⁾ who synthesised the acid portion by the following route:-



The obvious difficulty in this synthesis is the last stage which gives a mixture of isomeric olefins that had to be separated by fractional crystallisation of their N-(3,4-dimethoxybenzyl) amides. The resulting acid was converted to its acid chloride and condensed with vanillylamine to give a product sufficiently pure to be identified as capsaicin. An improved synthesis was finally due to Crombie and his co-workers.⁽¹¹⁾ The infrared spectrum of capsaicin

showed a band at 968.5 cm^{-1} attributed to a trans double bond. The required trans acid was therefore synthesised according to the following scheme:-



This method of synthesis of a trans double bond had already been used by Crombie and his co-workers to synthesise other unsaturated compounds, the evidence for the formation of a trans double bond being

the infra red adsorption. Ozonolysis of the intermediate alcohol gave isobutyraldehyde as expected; hydrogenation of the final acid gave the known, 8-methylnonanoic acid, thus proving its carbon skeleton. The resulting acid was converted to its acid chloride and condensed with vanillylamine to give a product identical (infra red spectrum and mixed melting point) with natural capsaicin.

This structure for capsaicin was generally accepted until the investigations of Kosuge et al.⁽¹²⁾ These workers showed, in a series of publications, that natural capsaicin could be separated, by paper chromatography into two components, only one of which contained an unsaturated side chain. It was shown that the olefin portion had the structure normally given to capsaicin and that the other component was dihydrocapsaicin, being identical to the compound obtained by hydrogenation of natural capsaicin. In addition to this, only the olefinic portion gave a dibromide and could be ozonised to isobutyric acid and adipic vanillylamide. Since this series of papers other workers⁽¹³⁾ have also reported similar results, they also being apparently unaware of this earlier work.

Our own investigations (see later) confirm the Japanese findings but show, however, that natural capsaicin is an even more complex mixture.

A brief survey of the biosynthesis of fatty acids and the aromatic acids will be given in the following sections. Attention will be

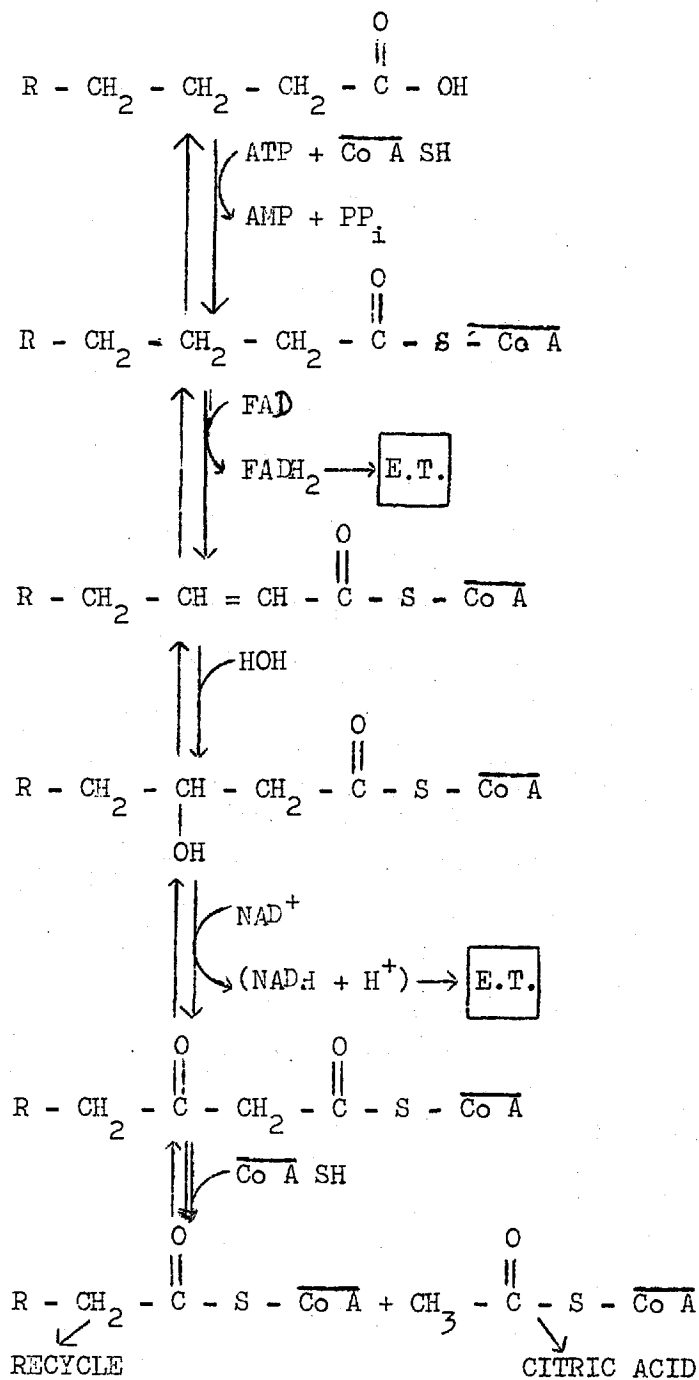
confined to those aspects which are relevant to a discussion of capsaicin biosynthesis. Work that has already been well reviewed will be summarised briefly with reference to the most recent review article.

The Biosynthesis of Fatty Acids

Evidence for the pathways of fatty acid biosynthesis^(14,15) in living organisms grew out of the knowledge of the catabolism of fatty acids. It was postulated by Knoop, at the beginning of this century, that fatty acids are degraded two carbon atoms at a time starting at the carboxyl end, by a process of "Beta oxidation". It was later shown that Coenzyme A (Co ASH) plays a key role, the acid being esterified with Coenzyme A, all subsequent steps involving acyl-Co A intermediates. The overall scheme was as follows:-

The following abbreviations are used:-

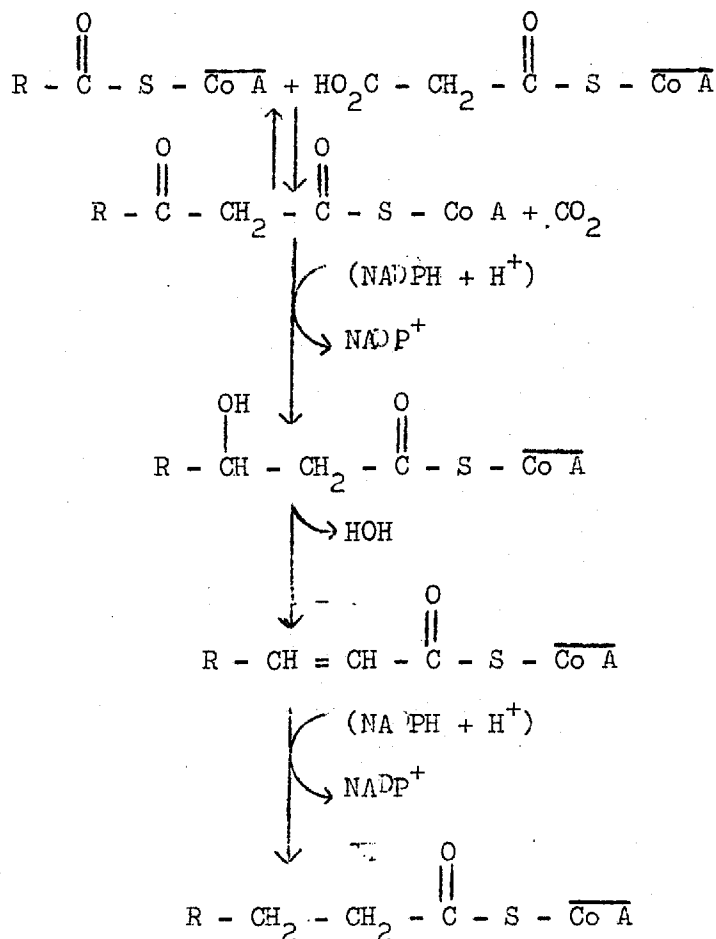
AMP	Adenosine mono-phosphate
ATP	Adenosine tri-phosphate
FAD	Flavin-adenine dinucleotide
FADH ₂	Flavin-adenine dinucleotide reduced form
NAD	Nicotinamide-adenine dinucleotide
NADH ₂	Nicotinamide-adenine dinucleotide reduced form
E.T.	Electron Transport System of the mitochondria
NADP	Nicotinamide-adenine dinucleotide phosphate
NADPH ₂	Nicotinamide-adenine dinucleotide phosphate reduced form
P _i	Inorganic orthophosphate
Co A	Coenzyme A
Co ASH	Reduced Co A



CYCLE

This process is repeated until the long-chain acyl group has been completely converted to acetyl Co A molecules. This identification of acetyl-Co A as the two carbon intermediate gave an indication of the mode of fatty acid biosynthesis, as the acetyl group is also the elemental building block in the formation of the long hydrocarbon chain. It was established that acetate, or molecules that could be converted into acetate, provided all the fatty acid carbon atoms, both atoms being incorporated in a head to tail relationship. It was therefore clear that biosynthesis could be achieved if the direction of degradation was reversed. Using an in vitro enzyme system which catalysed fatty acid formation from acetate alone, it was found that the formation of acetyl Co A required the presence of A.T.P., Co A SH, and NADPH as a reducing agent. Carbon dioxide was also needed, but it did not enter the final fatty acid structure. Later studies revealed the need for a biotin-containing enzyme in the system, which apparently catalysed the formation of malonyl-Co A in the presence of A.T.P., acetyl-Co A, and carbon dioxide. This process involved the formation of a CO_2 -biotin enzyme complex which then transferred the carbon dioxide to an acyl acceptor to give malonyl-Co A. The malonyl-Co A acts as a more effective two carbon donor than acetyl-Co A and provides the two "acetate carbons" at the carboxyl end of a saturated acyl group. The "malonyl carbon atom" reappears as free carbon dioxide, thus facilitating the reaction in the direction of synthesis. By means

of this condensation-decarboxylation reaction, the primary lengthening of the carbon chain is achieved:-



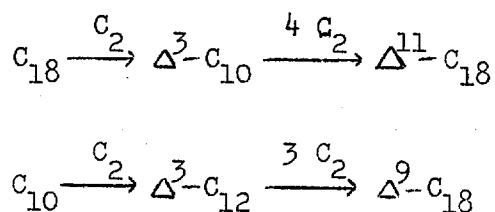
Further steps resemble the reversal of fatty acid oxidation. This cycle is repeated until the acid of desired chain length is obtained. For example, seven cycles are needed to give palmitic acid from acetyl-Co A.

Although it has been demonstrated that acetyl-Co A and malonyl-Co A are the primary substrates presented to the synthetase enzyme system, it has been shown that organic participants in the reaction sequence leading to synthesis are protein-bound rather than free acyl-Co A intermediates. This synthetase is pictured as a multi-enzyme complex of high molecular weight. Recently it has been observed that the acyl intermediates are combined with a heat-stable protein of low molecular weight (HSACP), which is a polypeptide containing a single sulphhydryl group. Thus acetyl-Co A and malonyl-Co A are converted to the acetyl and malonyl polypeptides before the final steps of condensation and decarboxylation take place. (16)

Formation of Unsaturated Fatty Acids

It has been shown^(17,18) that the production of unsaturated fatty acids requires the presence of NADPH and molecular oxygen, the effective substrate being the coenzyme A derivative of the fatty acid. This system appears to be normal for all oxygen requiring organisms. This process, called "oxidative desaturation", leads to the formation of long chain Δ^9 -cis-mono-enoic acids. Additional unsaturation can be incorporated by the same mechanism. The formation of polyunsaturated acids is also accompanied by chain lengthening.

Although this is the most common method of introducing unsaturation, other routes are known, for example, in the anaerobic bacteria mono-enoic acids are produced by β - γ dehydration of medium chain β -hydroxy-acids with subsequent chain elongation of the resulting 3-enoates:-



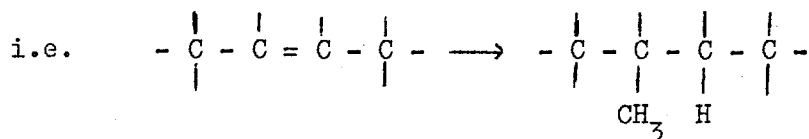
The mode of formation of trans double bonds does not seem to have been elucidated.

Branched Chain Fatty Acids

There are four known mechanisms which can lead to methyl-branched fatty acids⁽¹⁹⁾:-

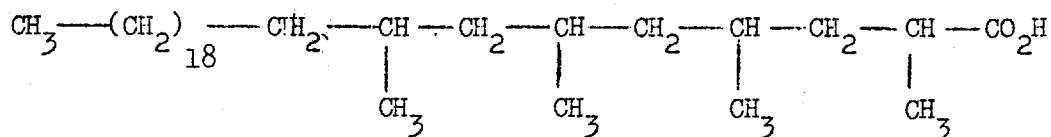
1) C-methylation with participation of L-methionine

This is well authenticated for numerous acids, for example, the formation of 10-methyl stearic acid from oleic acid. This involves addition to a double bond, and can also lead to the formation of cyclopropane rings which could open to give branched chains.



2) Incorporation of propionic acid

Fatty acids derived from propionic acid do not seem to have been found in higher plants. However, numerous examples exist in the animal kingdom, for example, the formation of mycocerosic acid from a C_{20} normal acid and four molecules of propionic acid,⁽²⁰⁾ and the



Mycocerosic acid

formation of α -methylbutyric acid and α -methylvaleric acid in the nematode ascaris lumbricoides.⁽²¹⁾

3) Incorporation of the branched chains of leucine and isoleucine

This route is principally used by bacteria as in the formation of the iso and anteiso acids (C_{15} - C_{17}) an example of this type being the formation of 12-methyl-tetradecanoic acid from leucine.⁽²²⁾

4) Incorporation of mevalonic acid

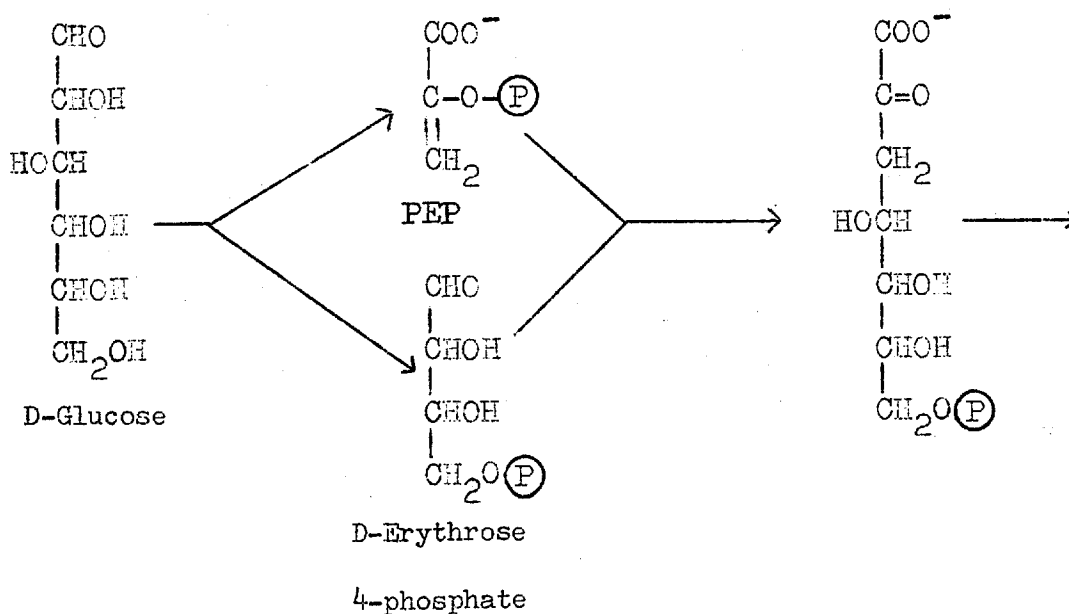
This contributes very little to the bulk of branch chain fatty

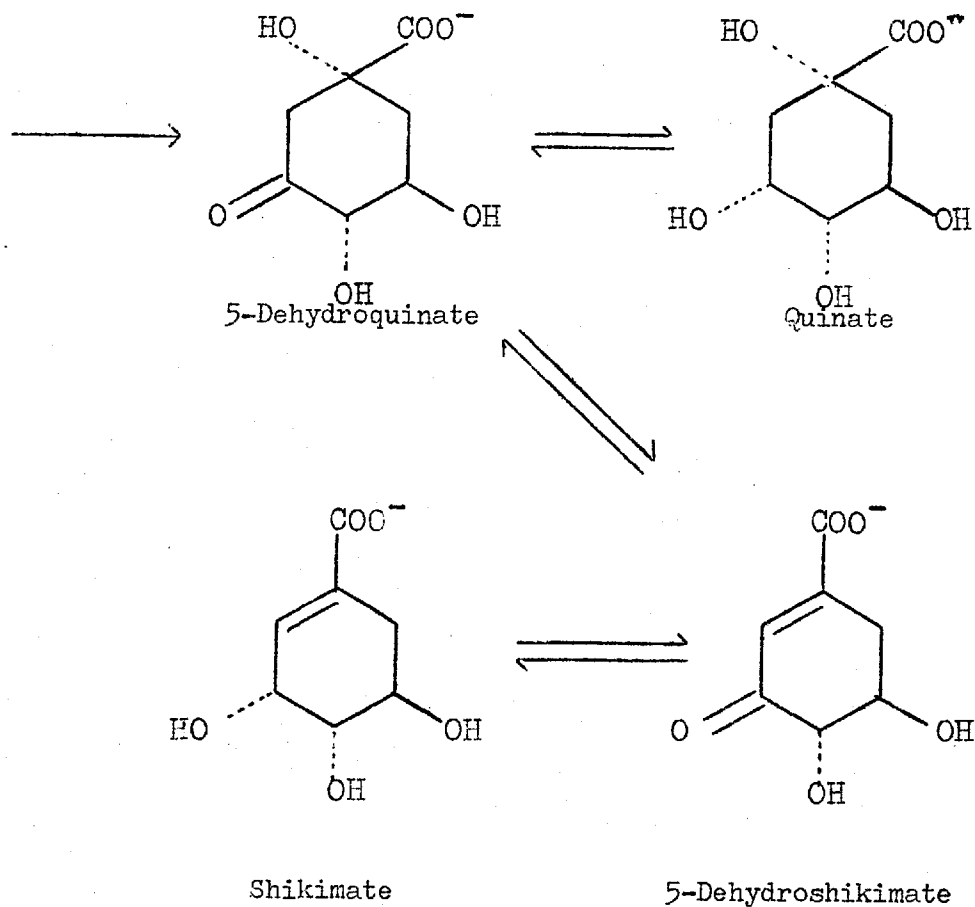
acids found in nature, notable exceptions being the apparently rare existence of 3,7,11,15-tetramethylhexadecanoic acid derived from phytol,⁽²³⁾ and the prenoic acids.⁽²⁴⁾

Aromatic Amino-acid Biosynthesis

The Shikimic Acid Pathway ⁽²⁵⁾

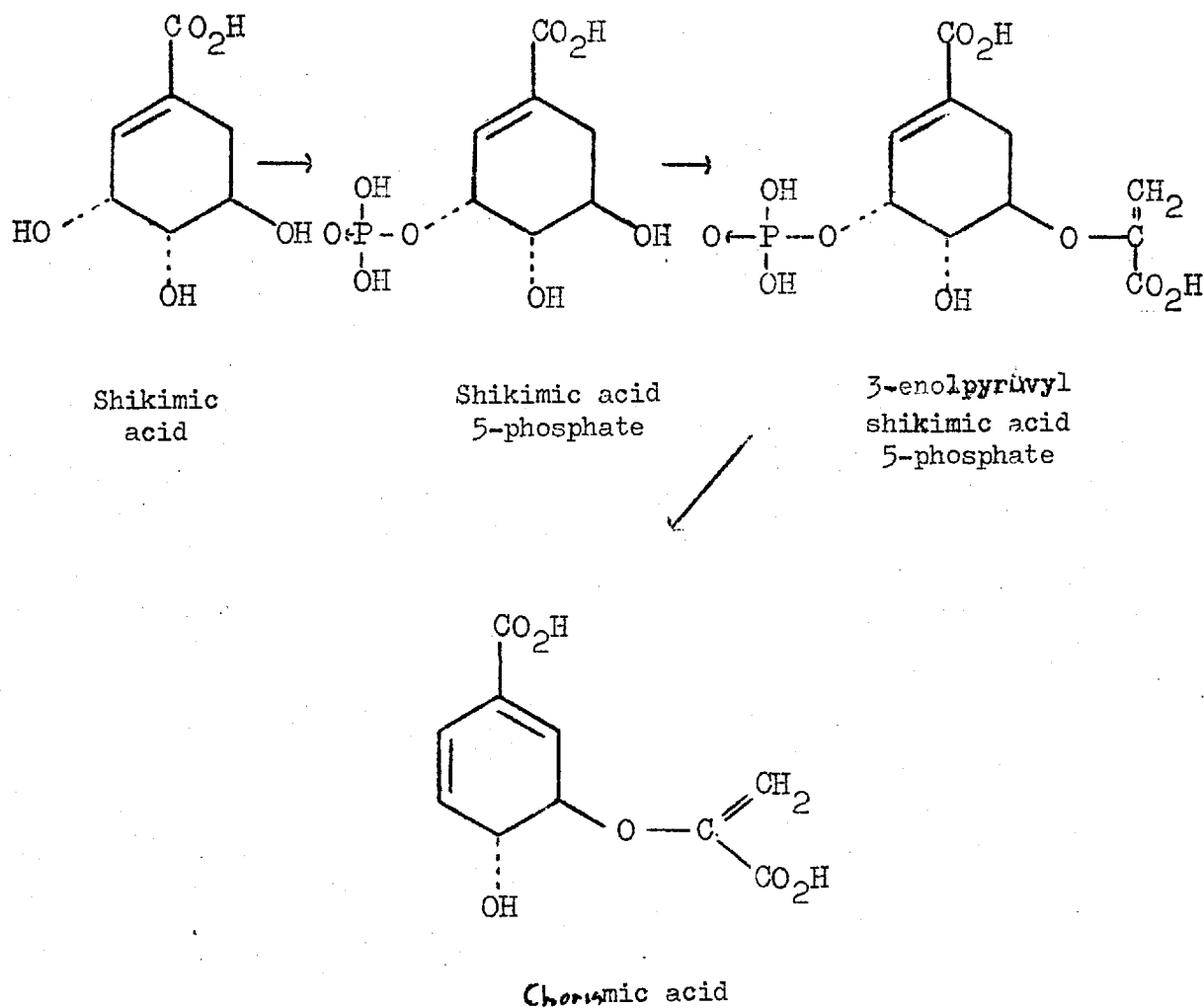
In higher plants there are a very large number and variety, of compounds biosynthetically related to the aromatic amino-acids and derived from the "shikimic acid pathway". This pathway uses phosphoenolpyruvate (PEP) and erythrose-4-phosphate and is thus closely connected with the pathway of sugar metabolism. The actual pathway for the formation of shikimic acid and the subsequent formation of aromatic amino acids was elucidated by using mutant organisms containing blocks in the pathway. These showed a need for certain precursors and also accumulated intermediates produced before this block. The pathway thus elucidated was as follows:-

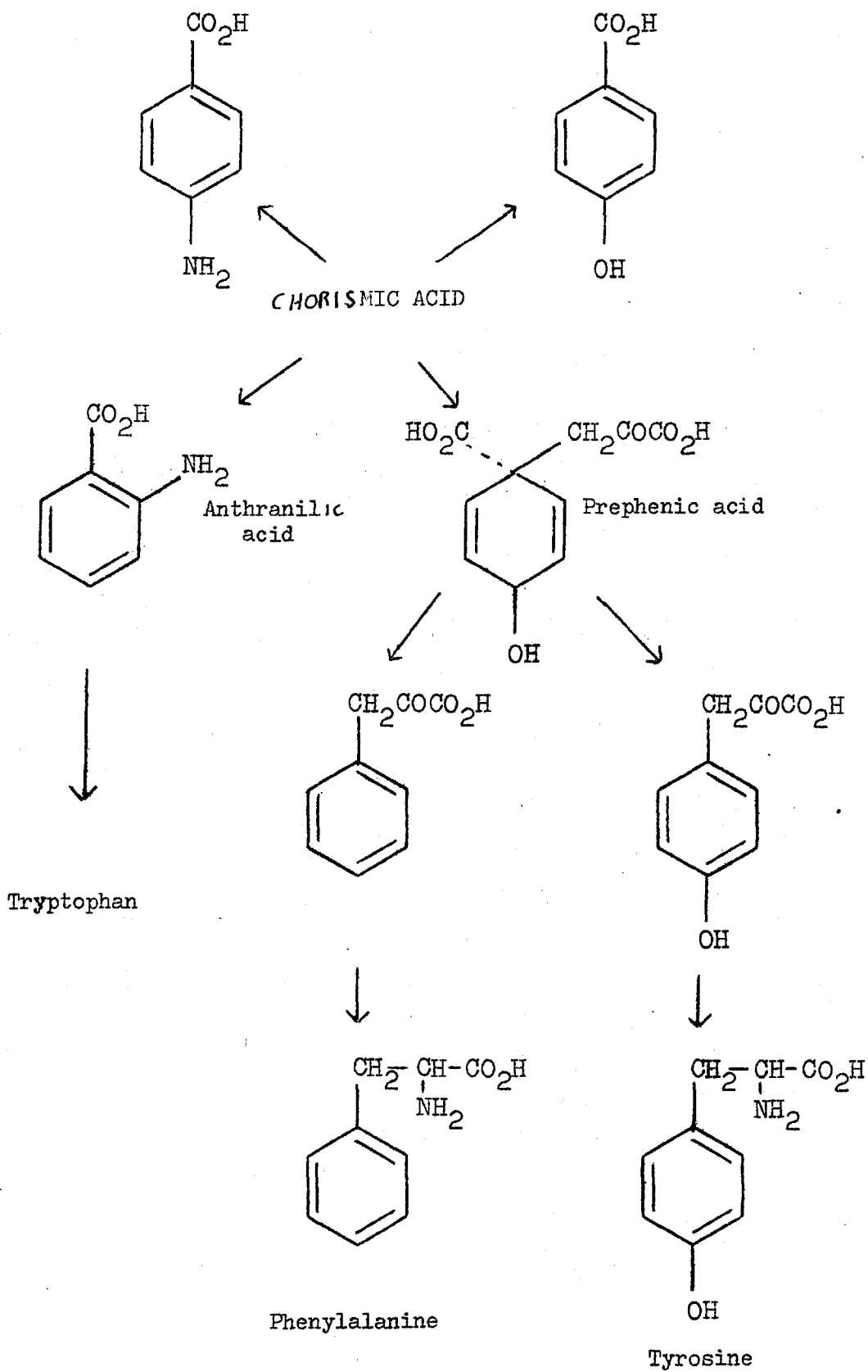




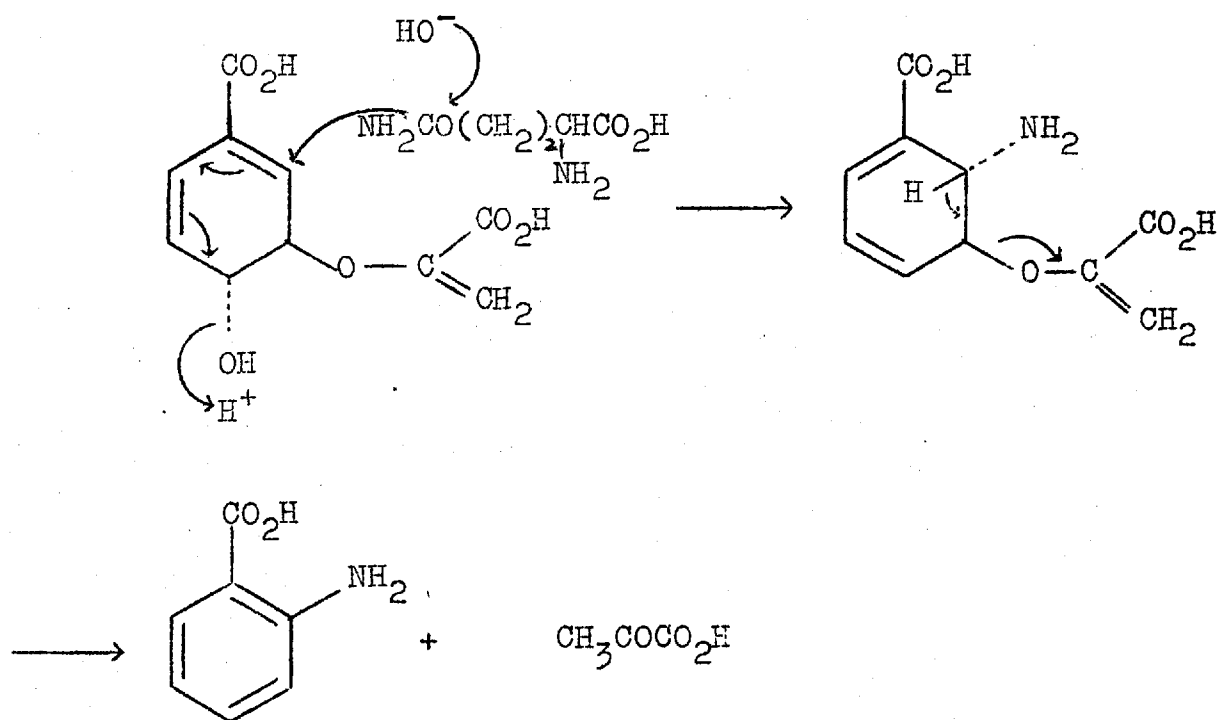
Shikimic acid was shown to be a common intermediate of many important aromatic compounds, including phenylalanine, tyrosine, p-hydroxybenzoic acid, p-aminobenzoic acid and anthranilic acid, the precursor of tryptophan. It was also known that prephenic acid was a precursor of both phenylalanine and tyrosine. Thus, various workers^(26,27) then searched for an intermediate lying between shikimic and prephenic acids. It was first shown that shikimic acid-5-phosphate and 3-enolpyruvyl-

shikimic acid were intermediates in the pathway leading to prephenic acid, and both were separately suggested as being the branch point leading to prephenic acid and anthranilic acid. Later evidence, again using mutant organisms, established that a new compound, chorismic acid, was in fact the key intermediate. The pathway thus elucidated was as follows:-





In the conversion of chorismic acid into anthranilic acid the amine group enters the 2-, and not the 6-position, of chorismic acid, no rearrangements of the ring taking place. (27,28)

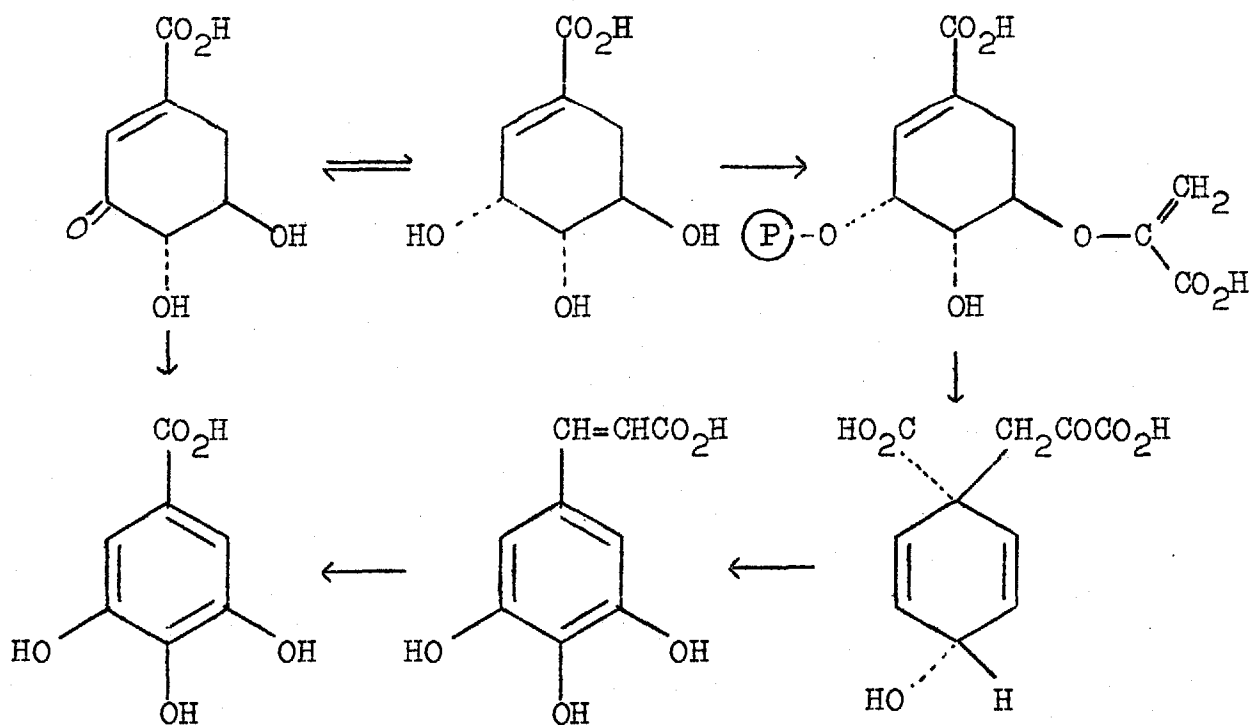


Anthranilic acid

The resulting anthranilic acid is the precursor of tryptophan.

It has also been confirmed that during the formation of l-tyrosine from shikimic acid no rearrangement of the ring takes place. (29) In addition to the above acids, evidence has been obtained that 5-dehydroshikimic acid is the precursor of protocatechuic acid (30) and

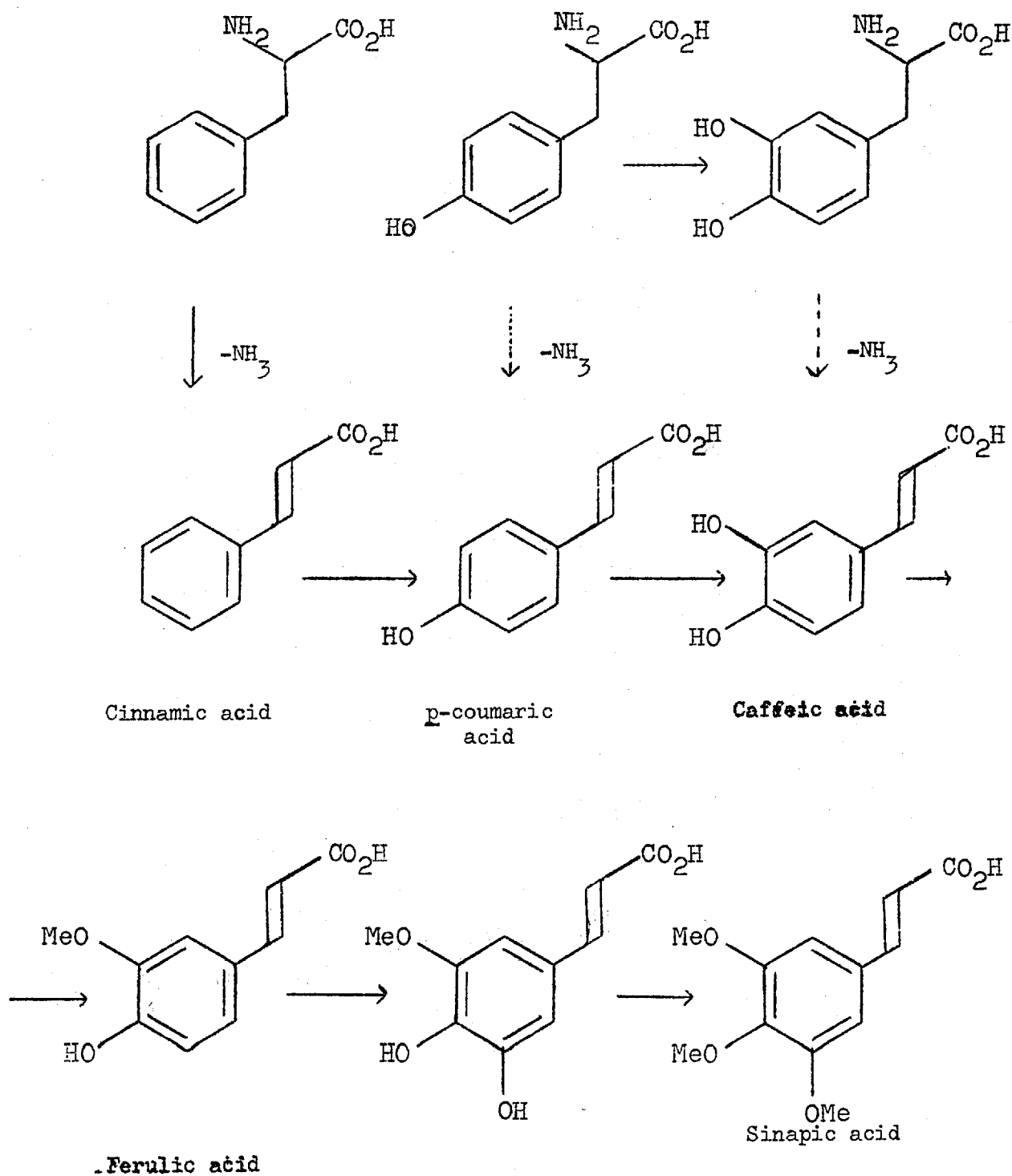
gallic acid.⁽³¹⁾ Two possible routes have been suggested for the formation of gallic acid:-



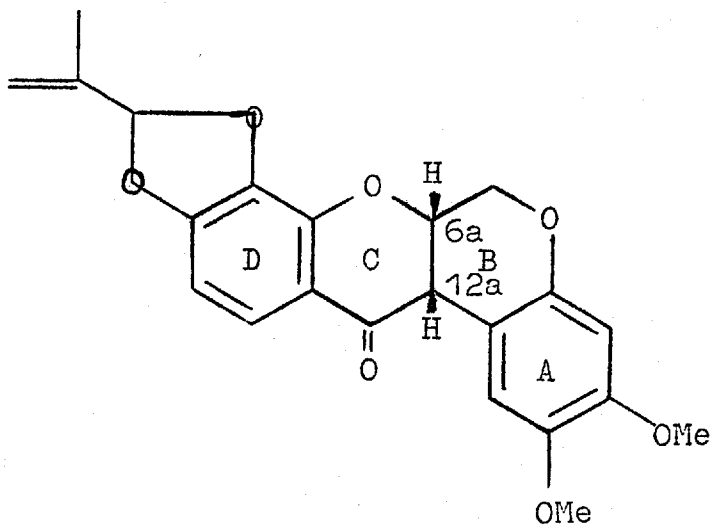
The relative importance of the above two routes has not yet been established although the direct route is favoured. A third mechanism for the formation of gallic acid has been found in Phyomyces blakeslanus in which the deamination of the amino-acid tyrosine is involved.⁽³²⁾ An analogous scheme has also been suggested for sumach (Rhus typhina) in which gallic acid is formed by β -oxidation of the C₃ side-chain of 3,4,5-trihydroxycinnamic acid.⁽³³⁾

The phenolic cinnamic acids formed in plants and converted into lignins are mainly derived from phenylalanine⁽³⁴⁾, the analogous

deamination of tyrosine to p-coumaric acid being only a minor route:-



The range of compounds derived from phenylalanine via the phenolic cinnamic acids is extensive. It includes, as previously described, the lignins, and also the flavanoids. A recent example, in which a rearrangement has taken place, is found in rotenone biosynthesis.⁽³⁵⁾ Here, a 1,2-aryl migration has taken place, similar to that found in the isoflavones. Thus ring A is derived from [2 - ¹⁴C]phenylalanine, the activity appearing at C_{12a}:-



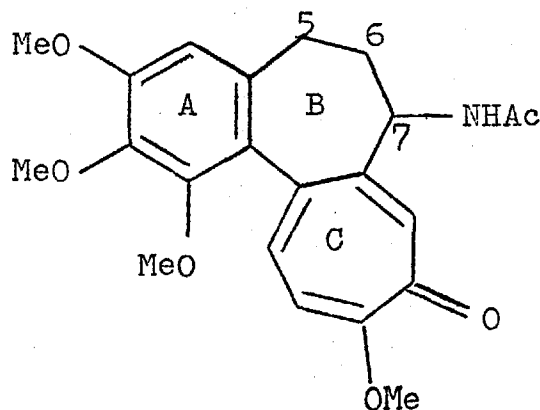
Rotenone

Biosynthesis of Nitrogen-containing Compounds derived from Phenylalanine
and Tyrosine

For the purpose of this topic it is convenient to cover only those nitrogen-containing compounds derived from phenylalanine and tyrosine and to classify them according to the proportion of the side-chain incorporated.

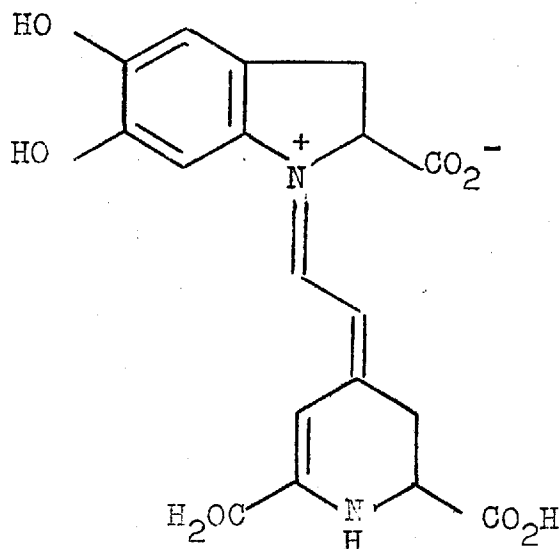
Compounds containing C₆-C₃ units

As previously described, phenylalanine is the precursor of the phenolic cinnamic acids involving loss of the amino-group. Another example of a compound incorporating the carbon skeleton of phenylalanine intact is colchicine^(36,37), in which ring A and C₅, C₆ and C₇ are derived from phenylalanine via cinnamic acid.



Colchicine

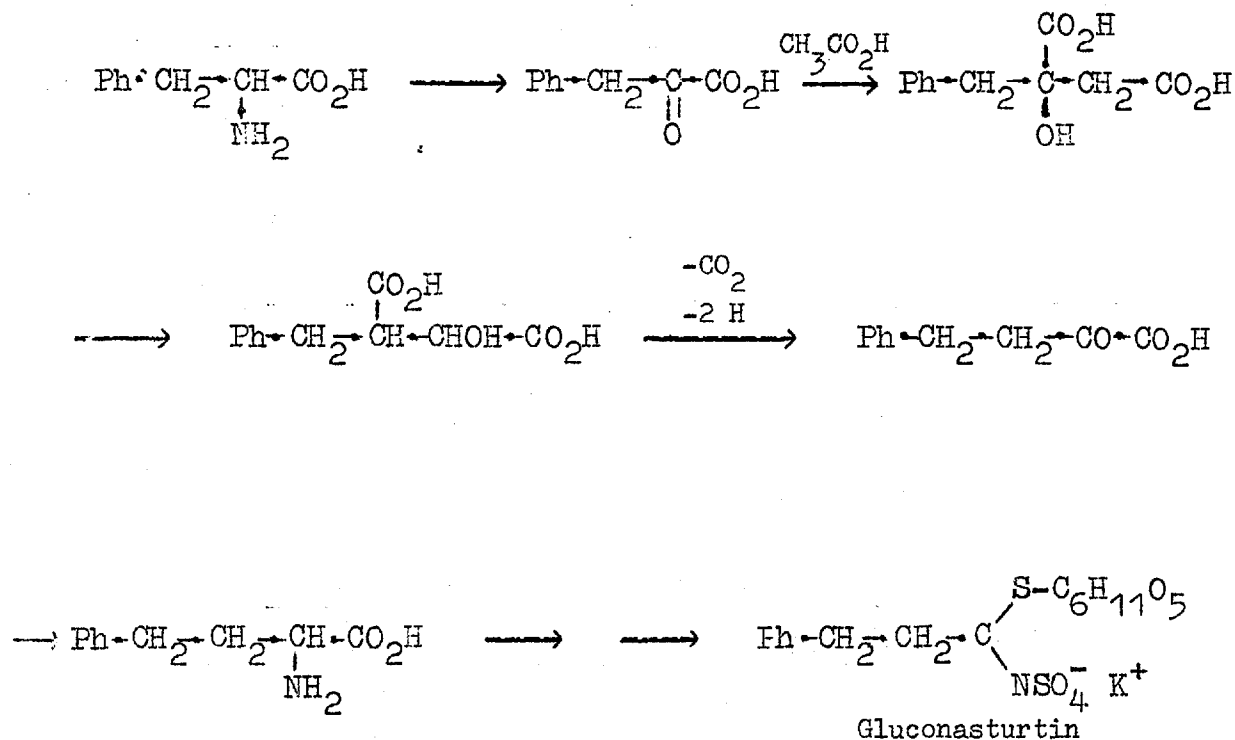
There appear to be very few compounds in this class derived from tyrosine, besides the polymeric melanins, only betanin⁽³⁸⁾, which is derived from 3,4-dihydroxyphenylalanine (dopa), has been reported.



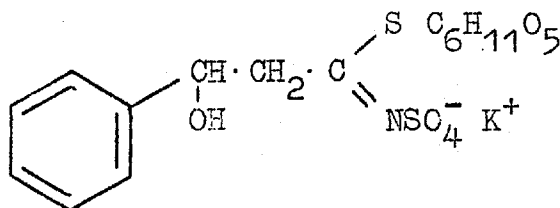
Betanin

Compounds containing C₆-C₂ units

The best-established examples of compounds of this class, derived from phenylalanine, are the mustard oil glucosides in which the rest of the side chain is derived from acetate units with the loss of the carboxyl group from phenylalanine, as follows⁽³⁹⁾:-

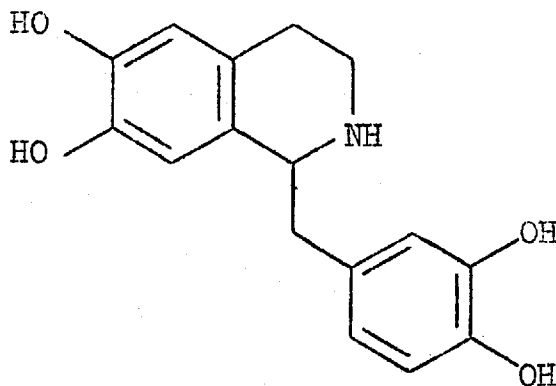


The intermediate amino acid, γ -phenylbutyrine, is the most effective precursor. A similar route is postulated for the formation of the mustard oil glucoside, glucobarbarin. (40)



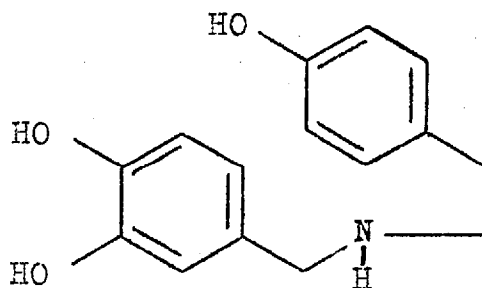
Glucobarbarin

Examples of nitrogen-containing compounds having C_6-C_2 units derived from tyrosine are numerous, including the papavarine/morphine series of isoquinoline alkaloids which are all derivatives of norlaudanosoline, derived from two molecules of tyrosine,



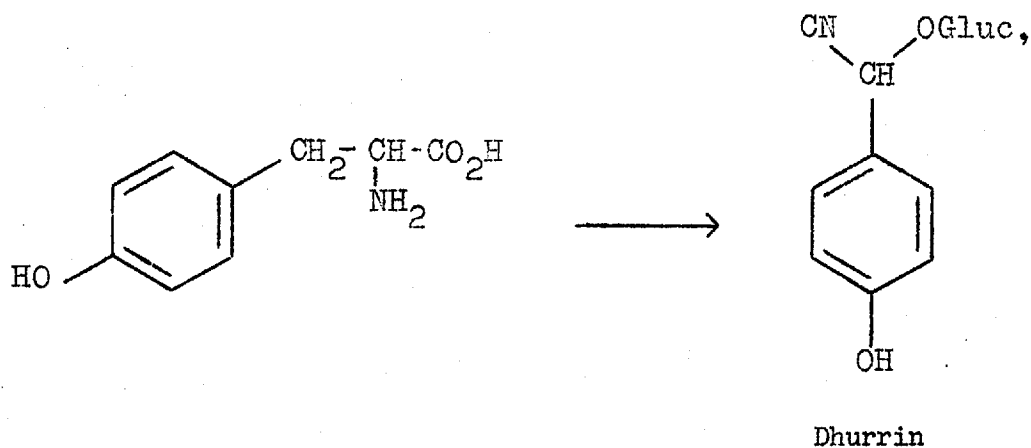
Norlaudanosoline

and the amaryllidaceae alkaloids,⁽⁴¹⁾ in which the parent base is norbelladine, derived from a 3,4-dihydroxybenzaldehyde unit and a tyramine unit.

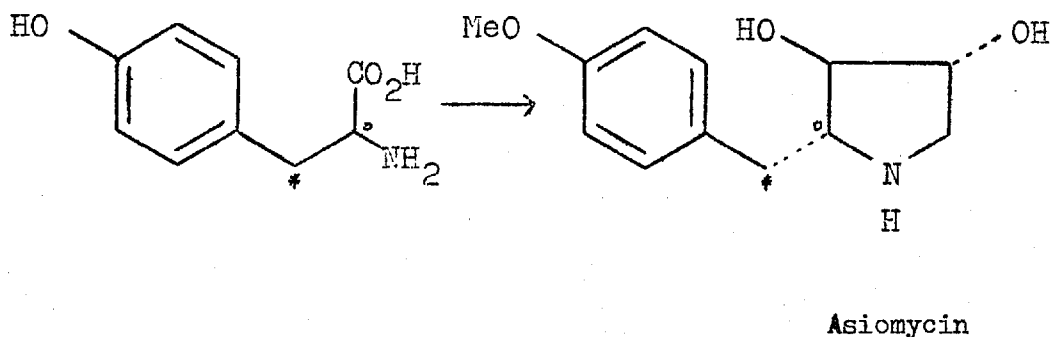


Norbelladine

In each case the alkaloids can be formed by oxidative coupling of the parent bases. An example of the mustard-oil glucoside type is dhurrin⁽⁴²⁾ in which the C_6-C_2-N unit of tyrosine is incorporated intact:-

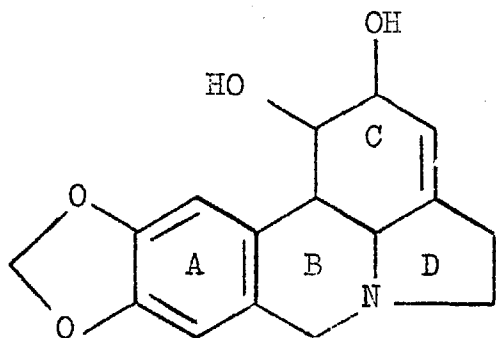


Finally, the alkaloid asiomyacin is derived from tyrosine and acetate units, the carboxyl group of tyrosine not appearing in the product. (43)

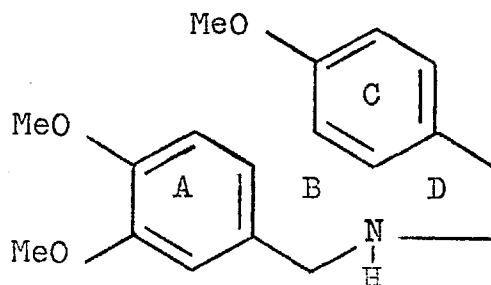


Compounds containing C₆-C₁ units

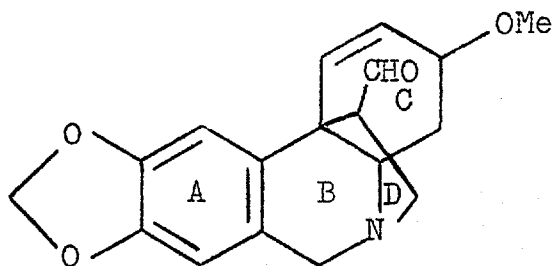
There are few examples of nitrogen-containing compounds with C₆-C₁ units known to be derived from phenylalanine. The best examples are to be found in the amaryllidaceae alkaloids, lycorine, belladine and haemanthamine,



Lycorine



Belladine

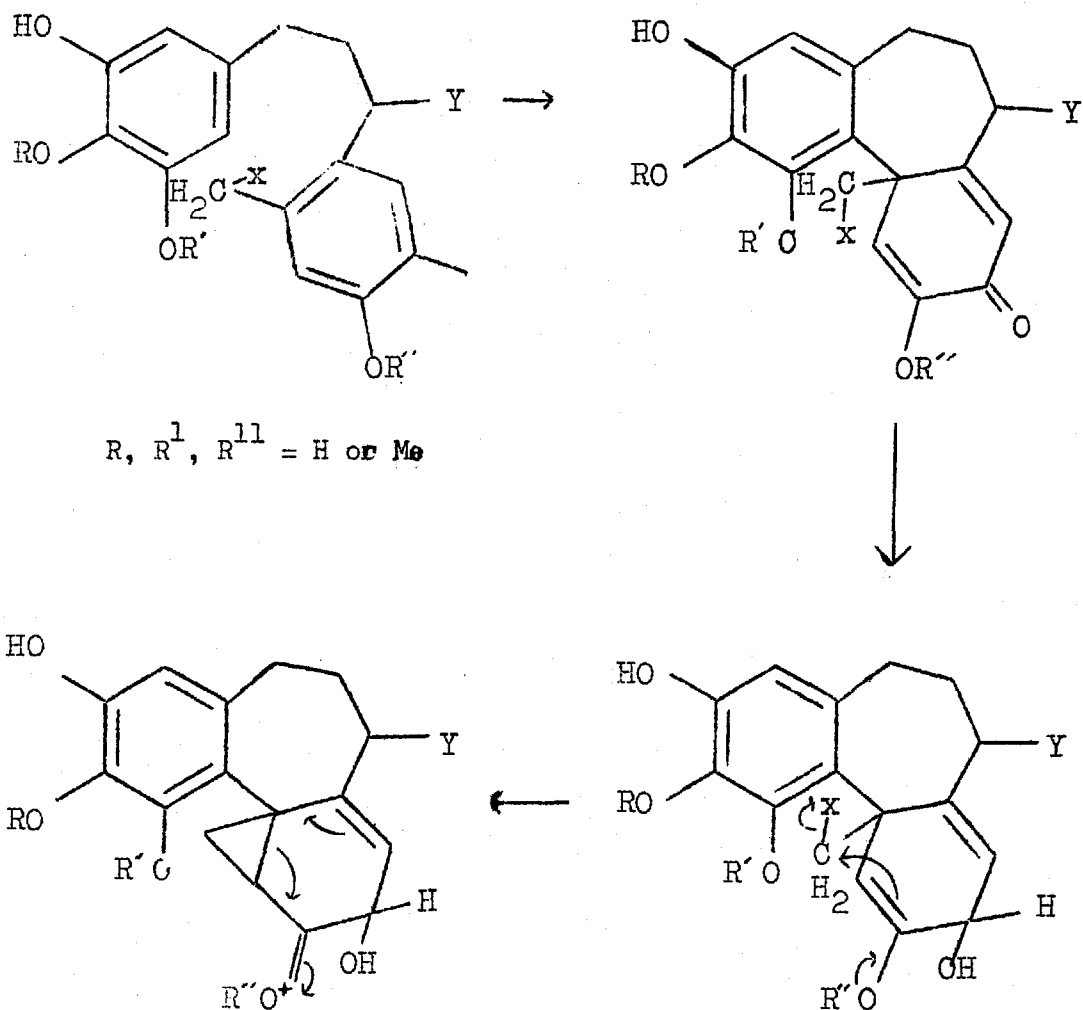


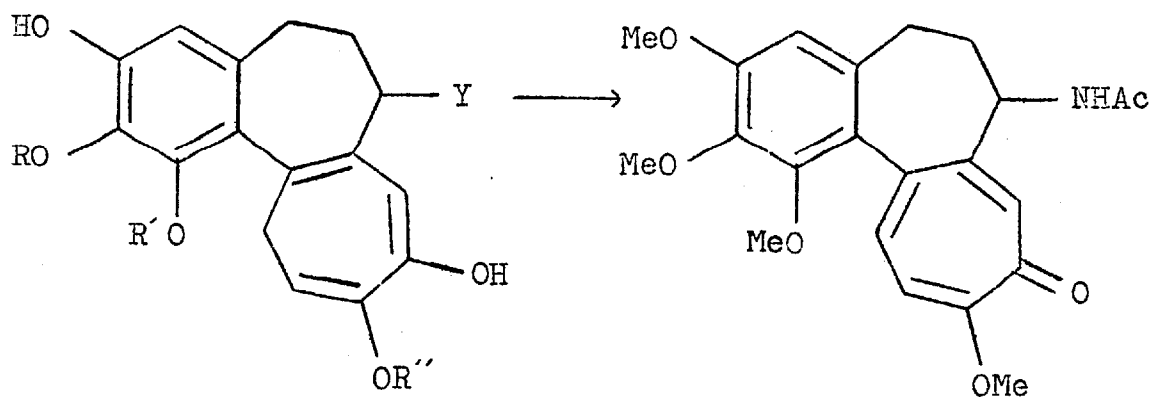
Haemanthamine

the parent base of this series being norbelladine which can be oxidatively coupled in various positions to give these structural types. In these alkaloids, rings C and D come from l-tyrosine, and ring A, with its benzylic carbon atom comes from phenylalanine via cinnamic acid. Thus protocatechuic aldehyde, caffeic acid, cinnamic acid and *p*-coumaric acid, are also precursors of the C_6-C_1 unit.⁽⁴¹⁾

The only nitrogen-containing compound with C_6-C_1 units derived from l-tyrosine which appears to have been reported is that of colchicine and its derivatives. The tropolone ring is derived from tyrosine; the rest of the molecule coming from phenylalanine. The

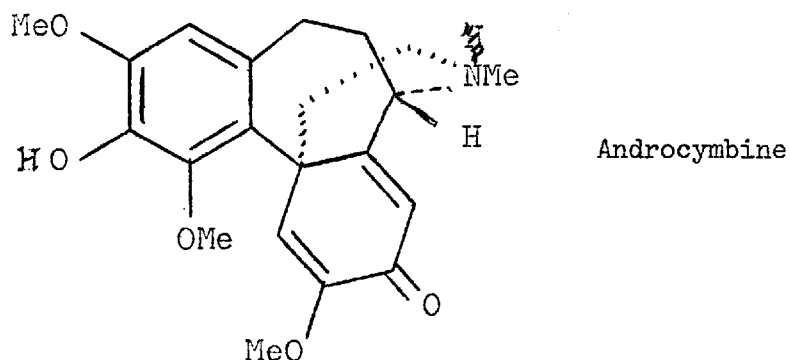
following mechanism has been proposed for its formation, the nature of x and y being unknown. (36,37,44)





Colchicine

However, the existence of androcymbine in the plant 'Androcymbium melanthioides' together with colchicine, 3-demethylcolchicine and N-formyldeacetylcolchicine, would suggest that tyrosine is first incorporated as a C_6-C_2 unit which is later degraded to a C_6-C_1 unit to give the tropolone ring.⁽⁴⁵⁾



The following conclusions concerning the origin of nitrogen-containing compounds derived from phenylalanine or tyrosine can be reached:-

- If the aromatic ring is not oxygenated then it is not derived from tyrosine, but can be derived from phenylalanine. If the ring is oxygenated it can be derived from phenylalanine or tyrosine.
- C_6-C_2 units come predominantly from tyrosine.
- C_6-C_3 and C_6-C_1 units, whether oxygenated or not, come predominantly from phenylalanine via cinnamic acid.

However, there are some exceptions to these generalisations, as have been previously described.

Clearly, the derivation of the C_6-C_1 unit of capsaicin cannot be predicted with confidence, and can only be decided by experiment.

The Chemistry of Capsaicin

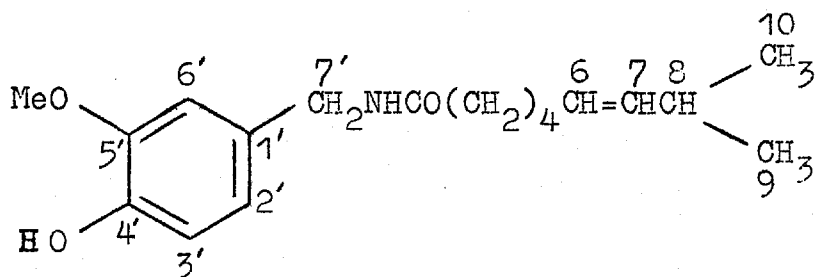
Capsaicin, isolated from *Capsicum oleoresin*⁽⁴³⁾, was obtained as a white crystalline solid, m.p. 64-5⁰, which, for reasons which will soon become apparent, will be called "natural capsaicin mixture". The n.m.r. spectra of natural capsaicin mixture and its derivatives showed only ca 1.5 protons in the olefinic region (τ 4.67) and an unexpected high field band at τ 9.2 beyond the methyl ~~doublet~~ of capsaicin olefin (Table 1). This seemed to suggest that there was some other compound present, probably dihydrocapsaicin. On comparing the n.m.r. spectrum with that of hydrogenated natural capsaicin mixture, it was clear that this was indeed possible. The methyl signal in the hydrogenated product occurred in the same position as the anomalous high field band (τ 9.2) of natural capsaicin mixture. In addition to this, accurate hydrogenation showed only 80% uptake of hydrogen, and, after ozonolysis of the O-methyl derivative (see later), there was a residue of a non-olefinic compound, the i.r. spectrum being essentially the same as that of O-methyl natural capsaicin mixture, but lacking any adsorption at 968 cm.⁻¹ attributed to the trans double bond.⁽¹¹⁾ The evidence suggested that natural capsaicin mixture contained at least two components, one being the dihydro derivative of the other, and methods for their separation were sought. Thin-layer chromatography on silica G and alumina plates, with various solvent systems, gave no

Table 1

N.m.r. Spectra of Capsaicin Derivatives in CDCl₃

(τ values listed, coupling constant J in c.p.s, s = singlet,

d = doublet, m = multiplet.)



Capsaicin

Compound	2', 3', 6'	6, 7	7'	OMe	Me (9, 10)
Natural Capsaicin Mixture	3.24 (3 H), m.	4.67 (1.45 H), m.	5.74 (2 H) d*, J 6.0	6.18 (3 H), s	9.05 (6 H) d, J 6.5; 9.2, s.
Dihydro Natural Capsaicin Mixture	3.22 (3 H), m.	---	5.69 (2 H), d, J 5.5.	6.18 (3 H), s.	9.14 (6 H), d, J 5.5.

Compound	2', 3', 6'	6, 7	7'	OMe	Me (9, 10)
<u>O</u> -Methyl Natural Capsaicin Mixture	3.15 (3 H), s.	4.56 (1.1 H), m.	5.61 (2 H), d, J 6.0	6.12 (6 H), s.	9.04 (6 H), d, J 6.5; 9.18, s.
<u>O</u> -Benzoyl Natural Capsaicin Mixture	1.7-2.0 (2 H) 2.3-7.3 (6 H), m.	4.68 (1.45 H), m.	5.64 (2 H), d, J 5.5	6.25 (3 H), s.	9.05 (6 H), d, J 6.5, 9.2 s
<u>O</u> -Methyl Capsaicin olefin fraction	3.21 (3 H), m.	4.6 (2 H), m.	5.67 (2 H), s.	6.13 (6 H), s.	9.05 (6 H), d, J 6.5.
<u>O</u> -Methyl Capsaicin dihydro fraction	3.20 (3 H), s.	—	5.66 (2 H), d, J 5.5	6.16 (6 H), s.	9.16 (6 H), d, J 5.5

Compound	2', 3', 6'	6, 7	7'	OMe	Me (9, 10)
Deuterated Natural Capsaicin	3.21 (2 H), s.	4.67 (1.5 H), m.	5.67 (2 H), d, J 6.0	6.19 (3 H), s.	9.05 (6 H) d, J 6.5; 9.19, ss.
Tetrabromo Natural Capsaicin Mixture	3.07 (1 H), s.	5.86 (1.56 H) m.	5.52 (2.4 H), d, J 6.0.	6.15 (3 H), s	9.03, m
Tribromo Natural Capsaicin Mixture	3.27 (1 H), m; 3.05 (1 H), m.	overlapping 5.6-5.95 (4 H), m.		6.15 (3 H), s.	9.03, m
Dibromo Dihydro Natural Capsaicin Mixture	3.07 (1 H) s.	---	5.54 (2 H) d, J 6.0	6.15 (3 H) s.	9.15 (6 H) d.

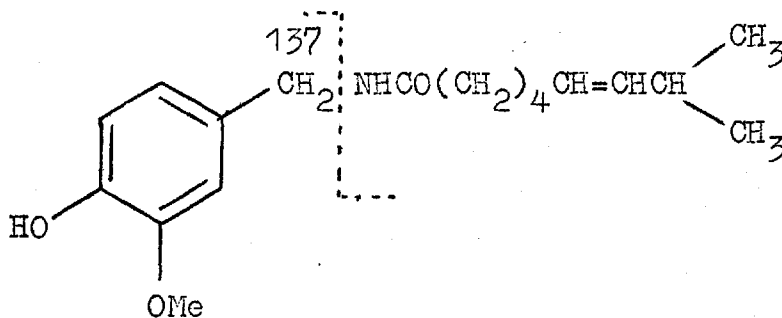
* Collapses to a singlet on shaking with D₂O.

indication of there being more than one component present. Therefore, since only one of the two components contained a double bond, methods for separating saturated and unsaturated compounds were investigated. This was finally achieved by thick-layer chromatography on Silica GF²⁵⁴ containing silver nitrate, a method described for the separation of the esters of naturally occurring unsaturated fatty acids.⁽⁴⁶⁾ This method gave two crystalline fractions from O-methyl natural capsaicin mixture. The faster running, minor component (ca. 25%), m.p. 75-6°, was a dihydro compound, O-methyl capsaicin dihydro-fraction, according to the i.r. and n.m.r. spectra (Table 1). The slower running, major product (ca. 75%) m.p. 75-6°, was the olefinic component, O-methyl capsaicin olefin-fraction, according to the i.r. and n.m.r. spectra (Table 1). The n.m.r. spectrum of the latter fraction now gave a good integration of the olefinic signal (2 H) and did not contain the anomalous high field band at τ 9.2 seen in the starting material. It also took up the predicted amount of hydrogen on catalytic hydrogenation. The n.m.r. spectrum of the dihydro portion was also identical in every way with the product obtained by hydrogenation of O-methyl natural capsaicin mixture. Similar results were obtained using the O-benzoyl derivative.

At this stage, a search of the literature revealed that some Japanese workers⁽¹²⁾ had obtained the same results on natural capsaicin mixture derived from Japanese Capsicum, the two components being separated by chromatography on cellulose powder. More recently,

other workers⁽¹³⁾, have reported the same findings using chromatography on polyamide to achieve the separation, being apparently unaware of the earlier Japanese work.

The mass spectrum of natural capsaicin mixture gave, besides the now expected molecular ion peaks at m/e 305 (M) due to capsaicin and at m/e 307 (M') due to dihydrocapsaicin, minor peaks at m/e 319 ($M + 14$), m/e 321 ($M' + 14$), and at m/e 293 ($M' - 14$) (Table 2) which appeared even when the running conditions (temperature and inlet system) were varied. Accurate mass measurements (Table 3) showed that the formulae corresponded to homologues of capsaicin and dihydrocapsaicin. The rest of the mass spectrum of natural capsaicin mixture corresponded to the following modes of breakdown: at the benzylic position to give the base peak at m/e 137



and β to the carbonyl group with transfer of a γ -hydrogen.

Table 2

Mass Spectra of Capsaicin Derivatives

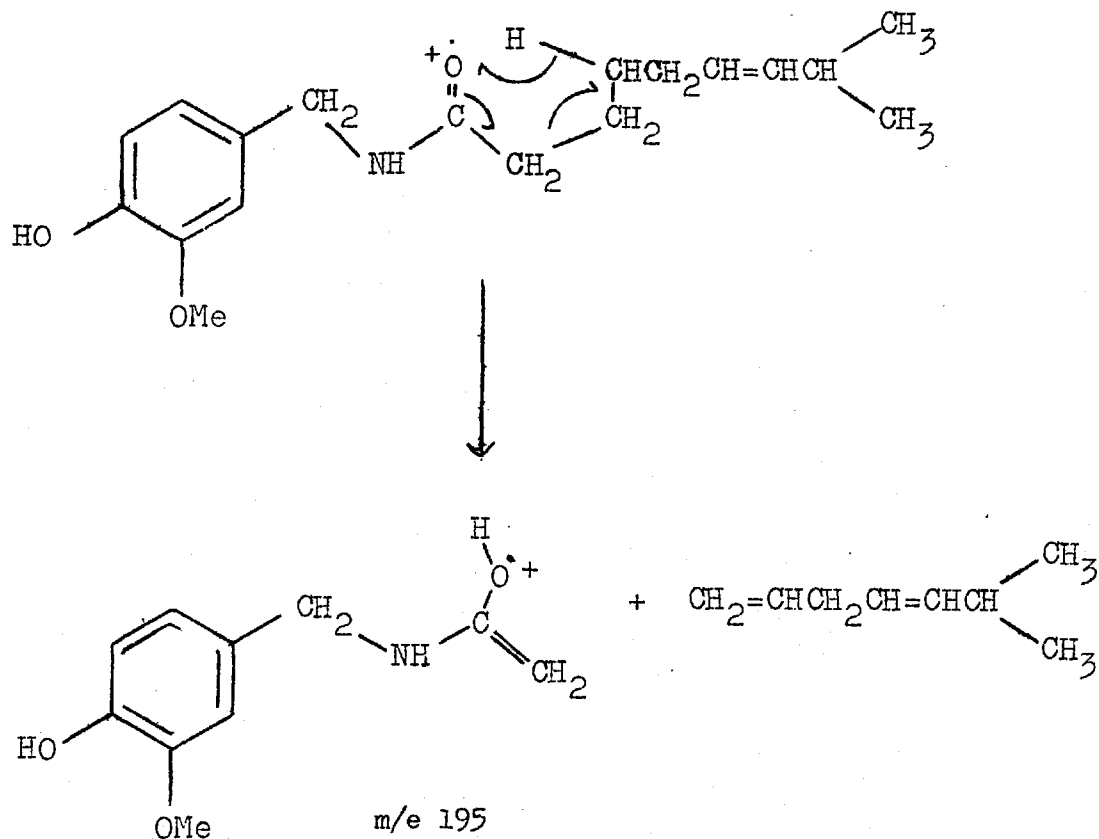
Compound	Molecular ion m/e	Relative intensities of Peaks		
		M	M + 14	M - 14
Natural Capsaicin	305	100	2.0	0
Mixture (a)	307	73.0	3.0	23.0
Hydrogenated Natural Capsaicin Mixture (b)	307	100	1.0	11.0
<u>O</u> -Methyl Capsaicin Dihydro-fraction (b)	321	100	5.0	20.0
<u>O</u> -Methyl Capsaicin olefin-fraction (b)	319	100	1	0
<u>N</u> -Veratryl Decanamide (c)	319	100	0	0

Compound	Molecular ion m/e	Relative intensities of Peaks		
		M	M + 14	M - 14
<u>N</u> -Vanillyl (b)	307	100	0	0
Decanamide (e)	307	100	0	0
Dihydro- <u>O</u> -Methyl Capsaicin olefin (d)	319	100	3	0
-fraction (b)	319	100	2	0
Ester derived from <u>O</u> -Benzyl Dihydro Natural Capsaicin Mixture (g)	378	100	5	8
Deuterated Natural Capsaicin Mixture	306	100	1	0
	308	35	1	9

(a) Heated Inlet System (H.I.S.), 170°. (b) H.I.S., 200°.

(c) H.I.S., 140°. (d) H.I.S., 165°. (e) Direct Inlet (D.I.), 100°.

(f) Probe 120°. (g) D.I., 120°.



Metastable peaks at the appropriate positions ($m/e \frac{195^2}{305}$ $m/e \frac{137^2}{305}$) confirmed these modes of fragmentation. Similar peaks were observed in the mass spectrum of O-methyl natural capsaicin mixture. The mass spectrum of O-methyl capsaicin olefin-fraction (Table 2) gave, besides the expected cracking pattern, molecular ion peak (M), and lack of any dihydro compound, a peak at $m/e \ 333$ ($M + 14$), there being no $M - 14$ peak. Accurate mass measurements (Table 3) gave the correct molecular formula for a higher homologue of O-methylcapsaicin. This compound was

Table 3

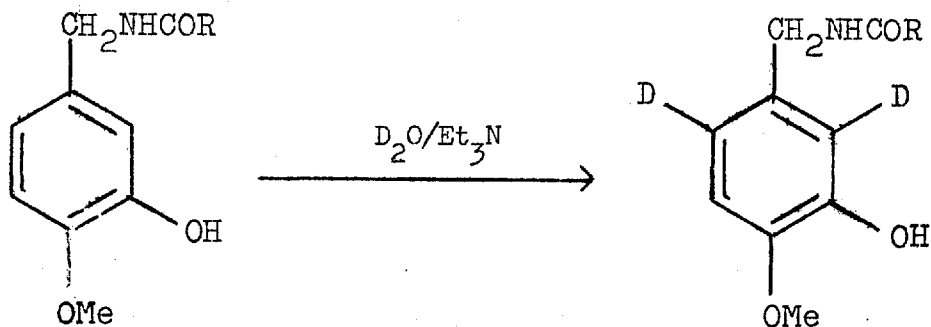
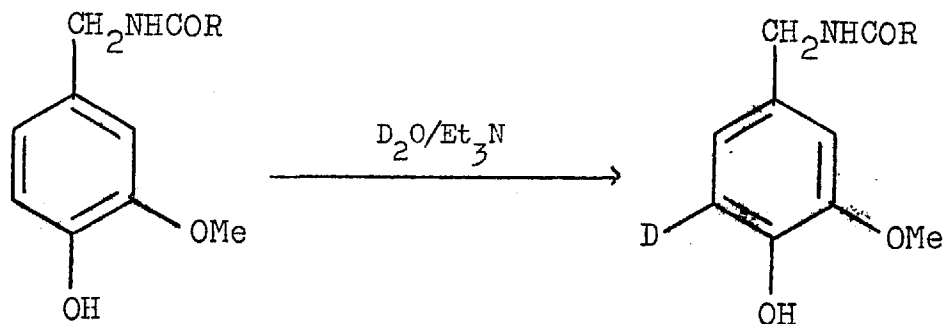
Molecular Formulae of Capsaicin "Homologues"

Derived from their Mass Spectra

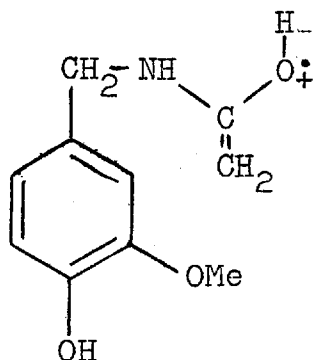
Compound	Fbund	Calculated	Molecular Formula
Natural Capsaicin	293.201	293.199	$C_{17}H_{27}NO_3$
Mixture	319.213	319.215	$C_{19}H_{29}NO_3$
	321.227	321.230	$C_{19}H_{31}NO_3$
<u>O</u> -Methylcapsaicin olefin-fraction	333.231	333.230	$C_{20}H_{31}NO_3$
<u>O</u> -Methylcapsaicin dihydro-fraction	335.245	335.246	$C_{20}H_{33}NO_3$

only present to the extent of 1% of the mixture as judged by the peak intensity. The mass spectrum of O-methyl capsaicin dihydro-fraction (Table 2) showed additional peaks at m/e 335 ($M + 14$, 1% of the whole), confirmed by accurate mass measurements (Table 3) to be a higher homologue of O-methyldihydrocapsaicin, and at m/e 307 ($M - 14$, 30% of the whole), confirmed by mass measurement to be a lower homologue. In order to discover whether these minor peaks represented true components of the mixture, the mass spectra of N-vanillyldecanamide and N-veratryldecanamide were also obtained under the same conditions (temperature and inlet system, Table 2). In neither were there any detectable $M - 14$ or $M + 14$ peaks. Furthermore, the mass spectrum of hydrogenated O-methyl capsaicin olefin-fraction showed only an $M + 14$ peak, and no $M - 14$ peak. Clearly then, the $M - 14$ peak from O-methyl capsaicin dihydro-fraction represents a lower homologue and not a fragmentation. Although the $M + 14$ peaks only occurred in natural capsaicin mixture and its derivatives, and not in the model compound, a recent report on the existence of $M + 14$ peaks in high molecular weight compounds⁽⁴⁷⁾ containing a basic nitrogen and a methyl source, such as $-CO_2Me$, suggested that there was still the remote possibility that methyl transfer might occur in capsaicin. Therefore capsaicin was degraded to see if the $M + 14$ peaks were also present in the mass spectra of the degradation products. The method used was that of thermal decomposition of a nitrosoamide to give the corresponding

ester⁽⁴⁸⁾. The O-benzyl derivative of dihydro natural capsaicin mixture was converted to the N-nitroso compound using nitrosyl chloride in acetic anhydride. The nitrosoamide was converted to the corresponding ester, O-benzylvanillyl 8-methylnonoate, on refluxing in carbon tetrachloride. This ester still showed the corresponding $M + 14$ and $M - 14$ peaks in its mass spectrum, and, since there was now no basic nitrogen in the molecule, this supports the evidence for these compounds being true homologues. Hydrolysis of the ester to O-benzylvanillyl alcohol and 8-methylnonanoic acid showed that the homologues were present in the acid portion of the molecule, there being an $M + 14$ peak only in the mass spectrum of the acid. Unfortunately, there was also no $M - 14$ component in the acid. This may have been lost during the isolation due to its greater volatility. Although, in capsaicin, the aromatic portion had the vanillin substitution pattern, there was clearly the possibility of there being an isomer present with the isovanillin substitution pattern. Thus, to investigate this possibility, natural capsaicin mixture was exchanged with deuterium oxide using triethylamine as a base. It is known⁽⁴⁹⁾ that phenols exchange only ortho and para protons under basic conditions, if any isovanillyl component were present this isomer would exchange in two positions, whereas the vanillyl component would exchange in only one,



and this would be evident in either the n.m.r. spectrum or the mass spectrum. The n.m.r. spectrum of the product showed that if any isovanillyl structure was present, then it was less than ca 10% of the whole, this being the limiting accuracy of the method. However, by comparing the $M : M + 1$ ratio of the fragmentation peak at m/e 195 in the mass spectra of natural capsaicin mixture and deuterated natural capsaicin mixture,



m/e 195

it was clear that, since the ratio was essentially the same in both cases, if any isovanillyl isomer was present it was only to the extent of less than 0.3%. Also, all the M , $M + 14$, $M - 14$ peaks in the mass spectrum of natural capsaicin mixture increased by one mass unit on deuteration indicating that these other components are also phenols and not O-methyl derivatives.

The ozonolysis of O-methyl natural capsaicin mixture was first investigated as a means of degrading capsaicin. However, on investigation by v.p.c., it was found that the steam volatile acid obtained using an oxidative work up ($\text{Ag}_2\text{O}/\text{NaOH}$ aq.) was a mixture of two compounds. Comparison by v.p.c. with all the known C_2 - C_5 acids showed that the expected acid, isobutyric acid, was the major product (ca 90%) and that the other acid was either isovaleric acid or active valeric acid, these being inseparable by v.p.c. In order to eliminate

the possibility that the valeric acid arose by some rearrangement of the ozonide, the model compounds, 2-methyl-8-phenyloct-3-ene and 2-methylhept-3-ene, were also ozonised, in neither case was there a trace of any but the expected steam volatile acids. Similar results were obtained by the oxidation of O-methyl natural capsaicin mixture using potassium permanganate in acetone. This evidence therefore suggests that there is an isomer in natural capsaicin mixture with the double bond in the 5-position and with the branched methyl group at C₇ or C₈.

Since natural capsaicin mixture had now been shown to contain both homologues, and isomers it was clear that the stereochemistry of the double bond should also be investigated. Previous evidence for the trans configuration relied on the i.r. adsorption at 968 cm.^{-1} , typical of a trans double bond⁽¹¹⁾. This clearly does not eliminate the possibility of the cis isomer also being present. The n.m.r. spectrum of natural capsaicin mixture in the olefinic region gives a complex multiplet which could not be interpreted as being due to a cis or trans double bond. Thus, in order to eliminate splitting due to the C₅ methylene protons and C₇ methine proton, spin-spin decoupling was tried on a 60 mc. n.m.r. spectrometer. Unfortunately, irradiating at the positions of the adjacent methine and methylene protons caused the olefin signal to collapse to a singlet which could not be resolved any further, thus giving no indication of the stereochemistry of the double

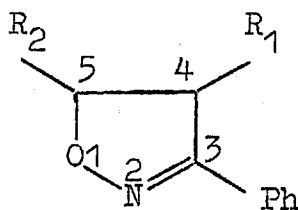
bond. Later investigations on an HA100 n.m.r. spectrometer gave, on spin-spin decoupling, the olefin signal greatly simplified but still as a multiplet which could not be interpreted to give the coupling constant with any confidence. Since the problem appeared to be due to the olefinic protons being in similar environments, the possibility of adding some reagent to the double bond which could put them in different environments was investigated. The method used was that of the 1, 3 dipolar addition of benzonitrile-N-oxide to an olefin to give a substituted Δ^2 -isoxazoline⁽⁵⁰⁾. Preliminary experiments using olefins of known stereochemistry showed that the two former olefinic protons would come in different regions of the n.m.r. spectrum (τ 6.5-7.0, τ 5.0-5.5), and that these regions were unoccupied in the n.m.r. spectrum of natural capsaicin mixture. These investigations also showed that there was an appreciable difference in the coupling constants of cis and trans protons (Table 4, J_{cis} ca 10 c.p.s., J_{trans} ca 6-8.5 c.p.s.). Unfortunately, no addition product of benzonitrile-N-oxide to natural capsaicin mixture, or its derivatives, could be obtained, and this method was therefore abandoned. This was probably due to some steric hindrance of the addition reaction as no addition product could be obtained from the model olefin, 2-methyl-8-phenyloct-3-ene. The last method of determining the configuration of the double bond involved the synthesis of a model olefin. The first compound investigated was 2-methylhept-3-ene which was prepared by the

Table 4

N.m.r. Spectra of Isoxazolines in CDCl₃.

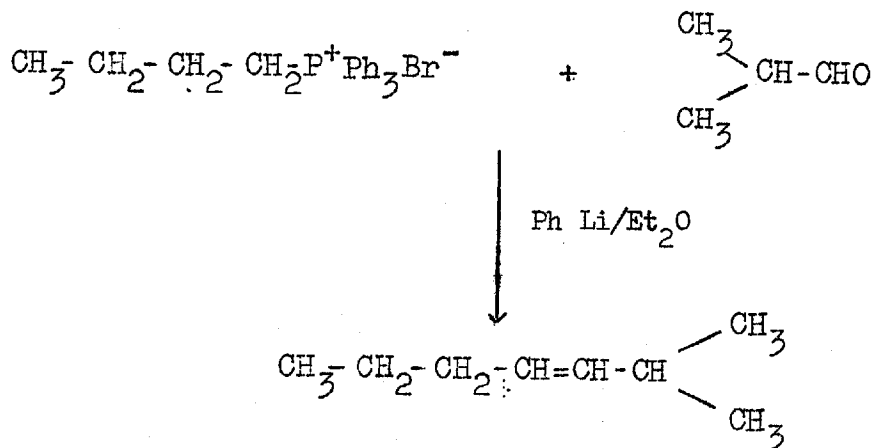
(τ values listed, coupling constant J in c.p.s., s = singlet

d = doublet, q = quartet, o = octet, m = multiplet)



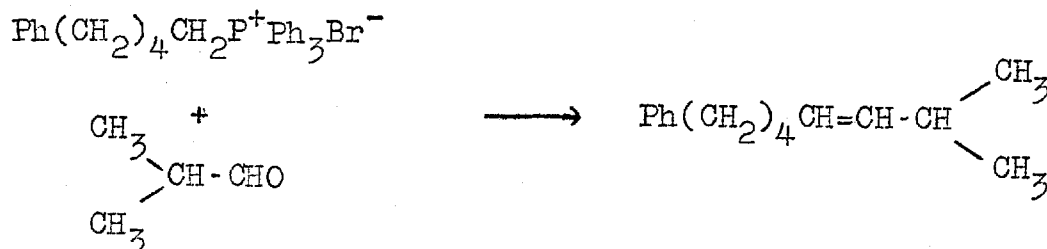
R_1	R_2	H_4	H_5	J_{45}
Ph	Ph	5.2, (d)	4.1 (d)	<u>cis</u> 9.5
Ph	Ph	5.35, (d)	4.5 (d)	<u>trans</u> 6.0
H	Ph	6-7, (o)	6.15-6.5 (q)	<u>trans</u> 10.6 <u>cis</u> 8.5
H	n-C ₆ H ₁₃	6.6-7.4 (o)	5.1-5.7 (m)	<u>trans</u> 10.0 <u>cis</u> 8.5

Wittig reaction between n-butytriphenylphosphonium bromide and isobutyraldehyde.



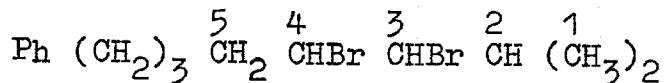
The product, purified by preparative vapour phase chromatography, gave an n.m.r. spectrum in which the olefin signal had a splitting pattern quite different from that of capsaicin. However, the configuration of this olefin was not known and methods for preparing olefins of known configuration were investigated. It was decided to prepare a corresponding acetylene which could then be selectively reduced by standard procedures to either a cis or a trans olefin. The starting material, 2-methylbut-1-yne, was prepared by bromination and dehydrobromination of 2-methylbut-1-ene. Unfortunately, the next stage, namely alkylation to give a di-alkyl acetylene, was unsuccessful even though a large variety of methods were tried. Since cis and trans olefins can be separated by t.l.c. on silver nitrate impregnated silica, the synthesis of an involatile model olefin was devised. Thus

2-methyl-8-phenyloct-3-ene was synthesised by the Wittig reaction, using 5-phenylpentyl bromide and isobutyraldehyde as starting materials. Initial attempts using phenyl-lithium as a base, in ether, gave only very poor yields of an impure product. Ultimately, on further investigation, the best yields (ca 60%) were obtained using potassium t-butoxide as a base in dimethylformamide. Since the latter solvent is polar, these conditions would tend to favour the cis compound. (51)



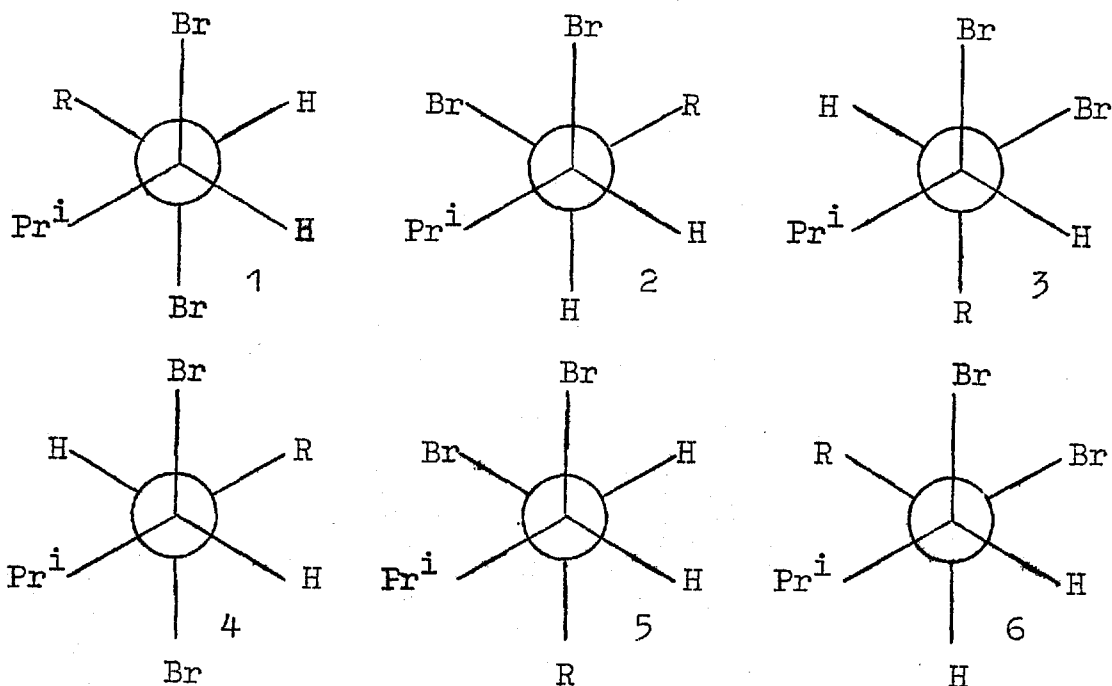
The product showed one spot on silica GF₂₅₄ plates containing silver nitrate, and was therefore either one isomer, or the isomers were inseparable by this method. The olefinic region of the n.m.r. spectrum showed a different splitting pattern to that found in natural capsaicin mixture. Attempts to influence the course of the Wittig reaction to give a trans product, using a non-polar solvent such as benzene, were unsuccessful, the product being the same as previously described and in much lower yields. The addition of a Lewis acid, namely lithium chloride, to the reaction, which is reported⁽⁵²⁾ to favour a cis product, gave the same product as previously obtained.

Since there was no definite proof of the configuration, attempts were made to convert the olefin to the corresponding acetylene which, could then be selectively reduced to the corresponding cis or trans olefin. The olefin was first converted to its dibromide by the addition of bromine in carbon tetrachloride at 0°. The n.m.r. spectrum of the product confirmed that it was the desired bromide and, also, the simple splitting pattern supported the presence of a single isomer probably existing in one major conformation. The coupling constants were determined using spin-spin decoupling on an HA100 n.m.r. spectrometer.



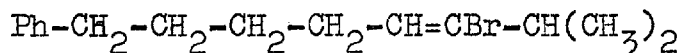
H ₁	τ 8.9	J ₂₃	7.9 c.p.s.
H ₃	τ 6.31	J ₃₄	2.6 c.p.s.
H ₄	τ 5.93	J ₄₅	6.5 c.p.s.

The following are the possible conformations viewed from the gem-dimethyl end along the C_3-C_4 bond, where $R = -(CH_2)_4Ph$.



Conformations 1, 2, and 3 would arise from trans addition of bromine to the cis olefin, and 4, 5, and 6 from trans addition to the trans olefin. Since J_{34} is small (2.6 c.p.s.) the dihedral angle between the protons at C - 3 and C - 4 cannot be near 180° . Therefore conformations 3 and 4 may be excluded. Structures 2, 5 and 6 are also unfavourable because of the close proximity of the two bulky, polar bromine atoms. The molecule appears then to exist mainly in conformation 1, which would clearly arise by trans addition of bromine to the cis olefin.

The next stage in the series, namely dehydrobromination using sodamide in liquid ammonia, gave a product which contained one olefinic proton as a triplet in its n.m.r. spectrum (τ 4.42). This suggests that the product was not the required acetylene but probably a vinyl bromide, only one molecule of HBr. being removed. The product is therefore probably 3-bromo-2-methyl-8-phenyloct-3-ene.



However, the model olefin was valuable for studies on the ozonolysis of natural capsaicin mixture (see above). This reaction was not pursued any further.

In conclusion, natural capsaicin mixture contains the following compounds: capsaicin (ca 74%), homocapsaicin (ca 1%), dihydrocapsaicin (ca 17%), homodihydrocapsaicin (ca 1%), and nordihydrocapsaicin (ca 17%). However, the proportions of the minor constituents rely on mass-spectral peak intensities which may be misleading. There is also evidence for the existence of an isomer of capsaicin with the double bond in a different position. The possibility of there being any cis isomer present has not yet been discounted.

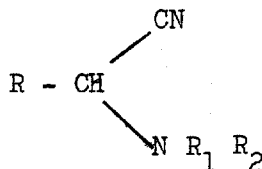
Synthesis of Precursors and Degradation of Natural Capsaicin Mixture

Aromatic precursors were labelled with tritium in such a position that the label in the derived natural capsaicin mixture would be ortho to the phenolic hydroxyl. The labelling pattern in the natural capsaicin mixture could then be determined very simply and unambiguously. Simple precursors such as sodium [$1\text{-}^{14}\text{C}$]acetate, sodium [$2\text{-}^{14}\text{C}$]acetate, and DL-[$2\text{-}^{14}\text{C}$]mevalonic acid lactone were purchased. The other precursors were synthesised as follows.

It was clear from the structure of capsaicin that it was probably derived from a simple amine, vanillylamine, and a branched chain fatty acid. Thus the first compound to be synthesised was vanillylamine. Vanillin was exchanged with tritiated water (1.8 mc./mmole. of exchangeable hydrogen) using triethylamine as a base, as had previously been described⁽⁴⁹⁾, to give [$5\text{-}^3\text{H}$]vanillin. This was converted to the corresponding oxime by a standard procedure. Various methods were tried for the reduction of the oxime to the amine, e.g. sodium amalgam, and lithium aluminium hydride with the oxime or the corresponding nitrile. The final method used was catalytic reduction of the oxime in the presence of hydrochloric acid, to prevent formation of the corresponding secondary amine. The amine salt so obtained, being insoluble in liquid scintillator, was diluted with inactive material and converted to the N-benzoyl derivative for counting.

The amine had an activity of 1.32 mc./mmole.

In order to establish whether the vanillylamine was incorporated into natural capsaicin mixture without degradation, a label at another position was needed to see if the ratio of the activity in the two positions was the same before, and after the feeding experiment. Experiments were made to incorporate tritium at the benzylic position (C - 7), starting from vanillin. Preliminary experiments using potassium cyanide as a catalyst to achieve exchange (via the cyanohydrin) of the aldehydic proton in vanillin, or O-benzyl vanillin, were unsuccessful, no aldehyde being recovered. Since the last stage in the preparation of vanillylamine was the hydrogenation of vanillin oxime, experiments designed to introduce tritium at this stage were investigated. Direct catalytic reduction of the oxime using deuterium gas, generated by dropping lithium metal into deuterium oxide, were successful. However tritium gas is relatively expensive and inconvenient to handle so an alternative was sought. The alkylation of the aminonitrile derivatives of aldehydes



using a strong base in various solvent media had been described in the

literature⁽⁵³⁾. Other investigations⁽⁵⁴⁾ showed that this alkylation could be achieved by generating the anion of the aminonitrile with sodium hydride in dimethylformamide.

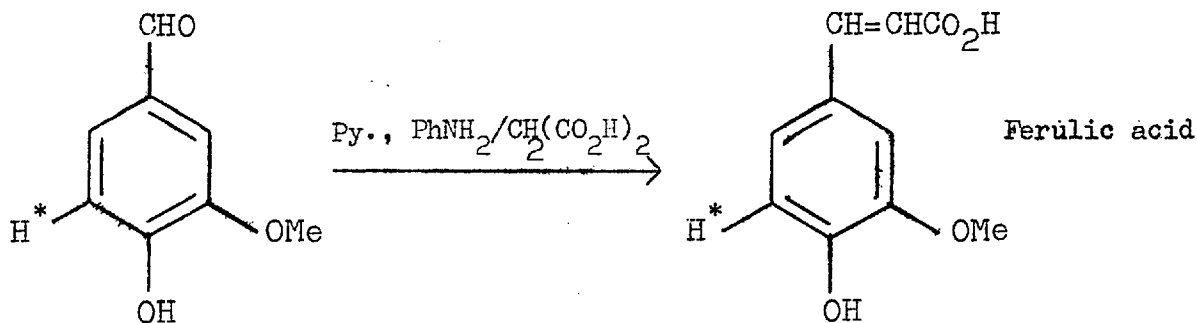
This reaction was therefore investigated as a means of incorporating tritium into the aldehydic position of vanillin or a simple derivative. Since vanillin has an acidic phenolic group, the O-benzyl derivative was used. The morpholinonitrile derivative of O-benzylvanillin was prepared using sodium cyanide and morpholine perchlorate in morpholine as a solvent. The anion was formed using sodium hydride in dimethylformamide, and was found to be stable under nitrogen. Exposure to air led to the slow formation of other products. The anion was quenched with deuterium oxide and excess carbon dioxide was passed through the solution to destroy excess base. The morpholinonitrile so obtained showed complete lack of the former aldehydic proton in the n.m.r. spectrum (τ 5.28). This deuterated morpholinonitrile was then hydrolysed with aqueous ethanolic hydrochloric to give the deuterated O-benzylvanillin. Its n.m.r. spectrum showed no aldehydic proton (τ 0.24), and the i.r. spectrum showed bands at $\nu_{\max}$ 2100, 2150, characteristic of a deuterated aldehyde. The aldehyde was converted to the oxime which was hydrogenated to the amine, the O-benzyl protecting group being removed by hydrogenolysis in this last stage. The product, 4-hydroxy-3-methoxyphenyl- $^{[2]}\text{H}$ methylamine hydrochloride, showed only one benzylic

proton (τ 6.07) in its n.m.r. spectrum. This series was then carried out using tritiated water (1.8 mc.) mmole. of exchangeable hydrogen) instead of deuterium oxide to give 4-hydroxy-3-methoxyphenyl- $[\text{}^3\text{H}]$ methylamine hydrochloride, with an activity of 1.47 mc./mmole.

L-Tyrosine was labelled in the 3, 5-positions by direct exchange of the di-sodium salt in tritiated water⁽⁵⁵⁾, giving an activity of 1.62 mc./mmole. Preliminary experiments on the conversion of L-tyrosine to phenylalanine⁽⁵⁶⁾ were only moderately successful, therefore DL-phenylalanine was completely synthesised as follows. The Grignard reagent from 3-bromotoluene was prepared and destroyed with tritiated water (1.8 mc./mmole of exchangeable hydrogen) to give $[\text{}^3\text{H}]$ toluene. As usual, a preliminary experiment with deuterium oxide was carried out. The $[\text{}^3\text{H}]$ toluene was then photo-brominated in the side-chain with bromine in carbon tetrachloride, under a tungsten lamp light source, to give $[\text{}^3\text{H}]$ benzyl bromide. This was condensed with diethyl acetamidomalonate, using sodium ethoxide in ethanol, and the product hydrolysed and decarboxylated to give DL-phenylalanine, which was purified by ion exchange chromatography to remove the glycine also formed. The product had an activity of 0.55 mc./mmole.

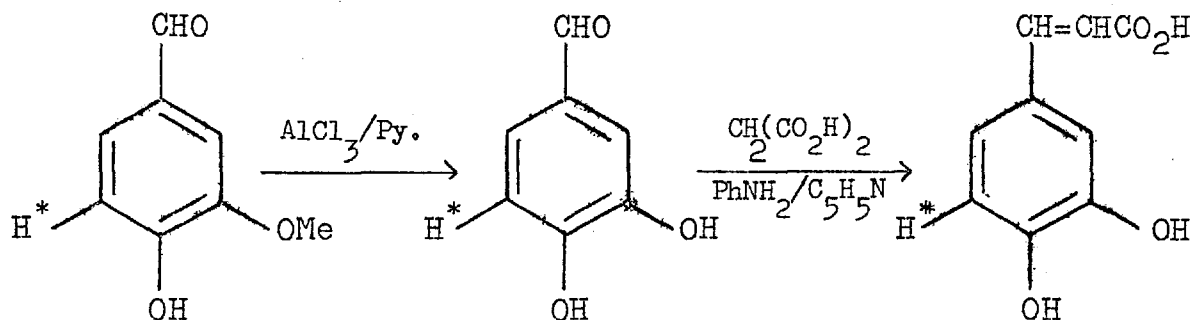
The results from feeding the aromatic amino-acids suggested that cinnamic acid and its derivatives should also be fed to Capsicum annuum and these acids were therefore prepared, again with tritium in the 5-position. Ferulic acid was prepared by the reaction of

[5-³H]vanillin, prepared as previously described, with malonic acid.

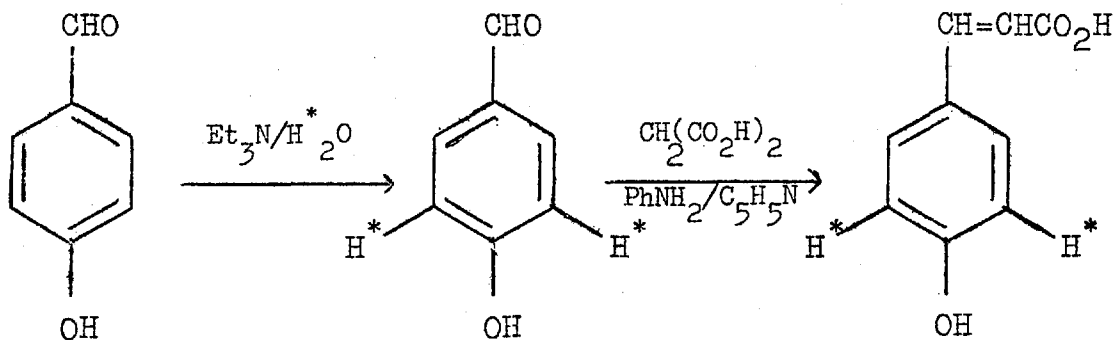


The ferulic acid obtained was counted by weighing out ca 1 mg., dissolving it in dimethylformamide (5 ml.), and taking 0.1 ml. aliquots which were diluted with liquid scintillator (1.1 ml.) for counting. The ferulic acid obtained had an activity of 1.37 mc./mmole. (from tritiated water, 1.8 mc./mmole. of exchangeable hydrogen).

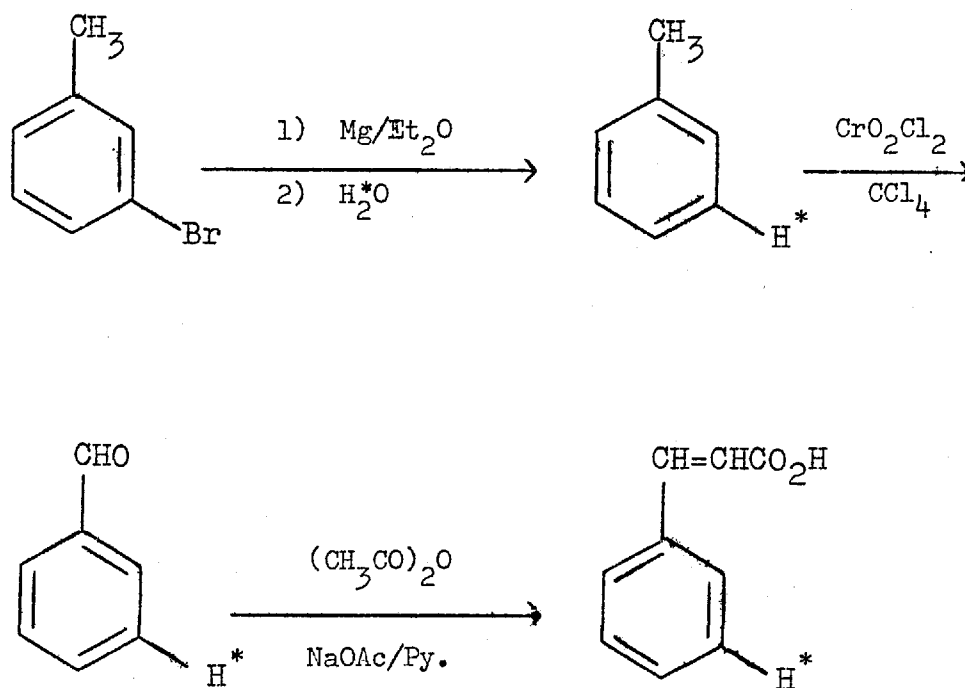
Caffeic acid was prepared by demethylation of [5-³H]vanillin, using aluminium trichloride and pyridine in dichloromethane, to give [5-³H]protocatechiuc aldehyde. This was then reacted with malonic acid as before to give tritiated caffeic acid



with an activity of 1.31 mc./mmole. The last of the substituted cinnamic acids, namely *p*-coumaric acid, was prepared from 4-hydroxy-[3- ^3H]benzaldehyde, the labelled aldehyde being prepared by direct exchange with tritiated water (1.8 mc./mmole. of exchangeable hydrogen) using triethylamine as a base.

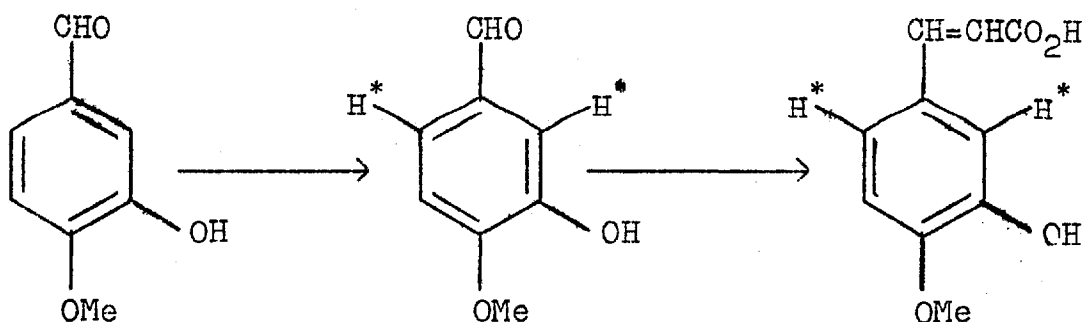


Cinnamic acid itself was prepared from [$3\text{-}^3\text{H}$]toluene (see above). Oxidation with chromyl chloride in carbon tetrachloride⁽⁵⁷⁾ gave [$3\text{-}^3\text{H}$]benzaldehyde. This was refluxed in acetic anhydride containing sodium acetate and pyridine, to give, after isolation, [$3\text{-}^3\text{H}$]phenylpropenoic acid with an activity of 1.52 mc./mmole.



After some of these compounds had been fed (see later), and most had been incorporated to some degree, the synthesis of a compound which should not be incorporated because of its substitution pattern, was

undertaken. The acid, hesperetic acid, was prepared as follows:-



Isovanillin was labelled with tritium in the 2-and 6-positions by direct exchange with tritiated water using triethylamine as a base.⁽⁴⁹⁾ The resulting [2, 6-³H₂]isovanillin was condensed with malonic acid in pyridine, containing a trace of piperidine, to give tritiated hesperetic acid.

As had been previously described, the majority of the aromatic precursors had been labelled with tritium in such a position (3 or 5 position) that the label in the derived natural capsaicin mixture would be ortho to the phenolic hydroxyl and should be easily removed by direct exchange with water. Preliminary experiments on [5'-³H] natural capsaicin mixture using both triethylamine or potassium t-butoxide as bases in water inexplicably gave only ca 50% exchange. Therefore the bromination of capsaicin as a means of removing the activity was investigated. Initial experiments on the monobromination of

N-vanillyldecanamide using one mole each of bromine and sodium acetate in acetic acid gave a product with a broad melting point ($68-72^{\circ}$ to 130°) indicating an impure compound. A similar result was obtained on treatment of natural capsaicin mixture with two moles each of bromine and sodium acetate in acetic acid. When dihydro natural capsaicin mixture was treated with one mole of bromine and sodium acetate in acetic acid, a mono bromo derivative was obtained with a sharp melting, and was analytically pure. The n.m.r. spectrum of the product showed two aromatic protons (τ 3.05, 3.29) with a fine splitting J of 2 c.p.s. indicating that they are meta to one another and that the bromine has entered the 5'-position (ortho to the phenolic hydroxyl), as would be expected. Monobromination of [5- 3 H] dihydro natural capsaicin mixture (labelled by exchange with tritiated water using triethylamine as a base) removed only 90% of the activity. Since it was possible that the natural capsaicin mixture was not only labelled in the 5-position of the aromatic ring, the synthesis of N-[5- 3 H] vanillyldecanamide was undertaken for further investigations of this bromination reaction. [5- 3 H]Vanillylamine hydrochloride (see above) was treated with n-decanoyl chloride and triethylamine in ether to give N-[5- 3 H] vanillyldecanamide. Monobromination of this amide gave an impure product with only ca 75% loss of activity. These results suggest that the position of substitution of bromine under these conditions is not exclusively in the 5-position (ortho to the phenolic hydroxyl group).

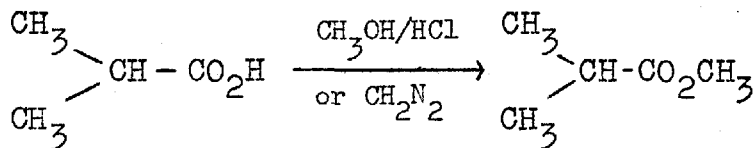
Since monobromination did not give a satisfactory result, the introduction of two bromine atoms into these compounds was next investigated. Treatment of N-vanillyldecanamide with two moles of bromine and sodium acetate in acetic acid gave a dibromo-derivative with a sharp melting point ($127-9^{\circ}$). The n.m.r. spectrum of the product showed only one aromatic proton ($\tau 3.07$), as required, but did not give any indication of the position of substitution of the second bromine atom. Similarly, dihydro natural capsaicin mixture gave a dibromo derivative with a similar n.m.r. spectrum in the aromatic region ($\tau 3.07$, 1 H). However, dibromination of both [5^1-^3H]dihydro natural capsaicin mixture and N-[$5-^3\text{H}$] vanillyldecanamide gave products with only ca 95% loss in activity. Since the position of substitution of the second bromine atom was not clear, investigations were carried out to determine this position. N-(Dibromovanillyl)decanamide was methylated using dimethyl sulphate and potassium carbonate in acetone⁽⁵⁸⁾ to give N-(dibromoveratryl)decanamide. Attempts were made to oxidise this compound directly to give a dibromoveratric acid, which could be compared with all the dibromoveratric acids. Unfortunately, this oxidation was unsuccessful. This investigation was then carried out in the reverse direction, namely by the synthesis of the expected product, N-(5,6-dibromovanillyl)decanamide, by an unambiguous route starting from vanillin. Vanillin was converted into 6-bromovanillin by bromination of vanillin acetate and hydrolysis of the product. Bromination of

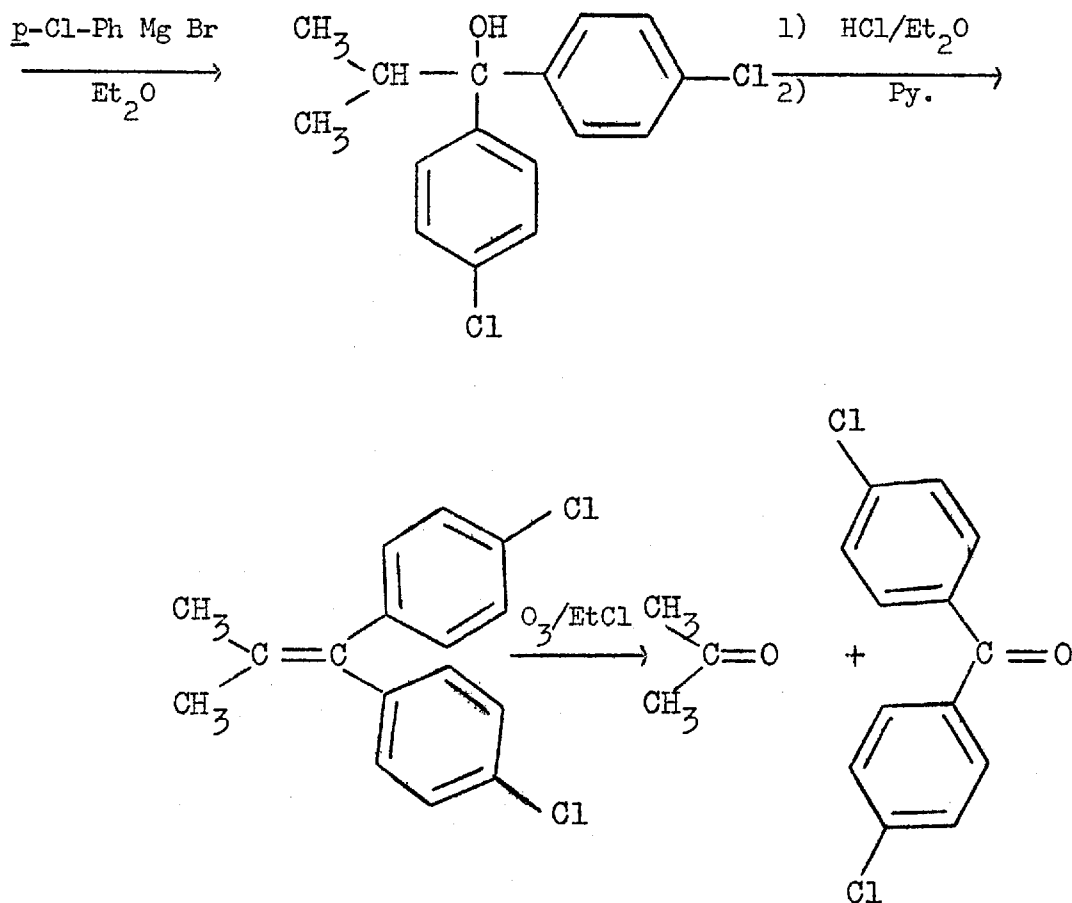
6-bromovanillin using bromine and sodium acetate in acetic acid gave 5,6-dibromovanillin which was then converted into the corresponding oxime. Attempts at preparing 5,6-dibromovanillylamine by the catalytic hydrogenation of 5,6-dibromovanillin oxime were unsuccessful, the product being vanillylamine (identical n.m.r. spectra), due to the loss of the bromine atoms by hydrogenolysis. Therefore this series was discontinued. Investigations were therefore continued into the removal of the activity from natural capsaicin mixture by direct exchange and, in view of the earlier failures to get complete exchange, more forcing conditions were investigated using the model compound, $N-[5-^3H]$ vanillyldecanamide, prepared as previously described. The effect of the mole proportion of triethylamine used was first investigated, at a temperature of 100° . These experiments showed that the best proportion to use was 0.5 mole. of triethylamine per 1 mole of natural capsaicin mixture, but even then only ca 88% exchange was obtained. The exchange was then repeated using 0.5 mole of triethylamine at a higher temperature of 118° with refluxing acetic acid as a heating medium. By this method a satisfactory exchange of ca 98% was obtained. This procedure was therefore used for degrading labelled natural capsaicin mixture obtained from the feeding experiments.

Since one of the precursors, 4-hydroxy-3-methoxyphenyl[3H]-methylamine hydrochloride, had tritium in the benzylic position and

should therefore give natural capsaicin mixture labelled in the corresponding position (at C - 7'), a mild method for breaking the amide linkage was needed. This degradation was also required for the investigation of the homologues in natural capsaicin mixture (see above). Existing methods resorted to the use of strong acids or bases at elevated temperatures.⁽⁵⁾ A search of the literature into the reactions of secondary amides, to see if any were useful as a means of splitting amide linkages, revealed that amides could be easily converted into their N-nitroso derivatives which could be rearranged under mild conditions to give the corresponding ester.⁽⁴⁸⁾ This method of degradation was first tried on the model compound N-veratryldecanamide. Treatment with one mole of nitrosyl chloride in acetic anhydride / acetic acid, containing potassium acetate, gave N-nitroso-N-veratryldecanamide as pale yellow crystals m.p. 33-4⁰. The infrared adsorption due to the N-nitrosoamide group appeared at 1720 cm.⁻¹. The N-nitrosoamide was then refluxed in carbon tetrachloride until all traces of the nitroso group were absent. The product obtained was the corresponding ester, veratryl decanoate, confirmed by its synthesis from veratryl alcohol and n-decanoyl chloride. (identical i.r. and n.m.r. spectra). These series of transformations were carried out on O-benzyldihydro natural capsaicin mixture as previously described to give ultimately the corresponding alcohol and acid portions, by hydrolysis of the ester.

As a first stage in the complete degradation of natural capsaicin mixture, obtained from the sodium $[1-^{14}\text{C}]$ acetate and sodium $[2-^{14}\text{C}]$ acetate feedings, the ozonolysis of natural capsaicin mixture was investigated. The ozonolysis was tried in various solvent media and at various temperatures, the reaction being followed by u.v. absorption of the product to check for degradation of the aromatic portion. Even under the mildest conditions tried, namely in ethyl chloride at -20° using an oxidative work up, only low yields (ca 20%) of isobutyric acid were obtained. Attempts to improve on this by using other methods of oxidation, eg. potassium permanganate in acetone, were unsuccessful, only the same low yield being obtained. The degradation of isobutyric acid, successfully carried out on inactive materials, was as follows:-





Methyl isobutyrate was treated with the Grignard reagent from p-chlorobromobenzene, and the product, 1,1-di(4-chlorophenyl)-2-methylpropan-1-ol, was dehydrated to the corresponding olefin, which was ozonised to give acetone and 4,4'-dichlorobenzophenone. The acetone could then be further degraded by standard techniques. This series was first carried out using the Grignard reagent derived from bromobenzene

but, since the intermediates were all liquids, the p-chloro derivative was used instead.

Biosynthesis of Capsaicin

All the precursors, in water (ca 5 ml.) were fed by injection into the young green seed pods (typically 12 pods) of Capsicum annuum at the rate of 0.1 ml. of solution per pod a day, for three days. The plants were left for a week after the feedings were complete. The pods were removed, ground up under liquid nitrogen, and extracted with acetone. The acetone soluble extract was dissolved in aqueous sodium hydroxide solution. This solution was extracted with ether, neutralised with carbon dioxide, and again extracted with ether to give the total phenols. The total phenols were diluted with natural capsaicin mixture (30 mg.) and chromatographed on alumina (Grade V) to give crude natural capsaicin mixture as a deep red oil. This was crystallised from di-isopropyl ether (with charcoaling) to give natural capsaicin mixture as a white crystalline solid, m.p. 64-5^o. The product was crystallised to constant activity. This was regarded as being constant when three consecutive crystallisations gave less than a 10% variation in activity. As a further check on its purity, a small portion (ca 5-10 mg.) was converted to the O-methyl derivative using dimethyl sulphate in aqueous sodium hydroxide. This then had the same "specific activity" as the natural capsaicin mixture. The incorporations of the aromatic precursors are given in the following table:-

Table 5

Precursor	Incorporation into Total Phenols	Incorporation into Natural Capsaicin Mixture	Activity left after exchange
[5- ³ H]Vanillylamine Hydrochloride	2.9%	0.05%	0.3%
4-Hydroxy-3-methoxy [5- ³ H]phenyl [³ H]methylanine Hydrochloride	2.5%	0.30%	—
DL-[3- ³ H]Phenylalanine I II	0.93% 1.19%	0.20% 0.50%	— 1.0%
L-[3- ³ H]Tyrosine I II	0.53% 1.33%	0.015% 0.014%	— 7%
3-(4-Hydroxy-3-methoxy [5- ³ H]phenyl)propenoic Acid (Ferulic Acid)	1.47%	0.026%	—

Precursor	Incorporation into Total Phenols	Incorporation into Natural Capsaicin Mixture	Activity left after exchange
3-(3,4-Dihydroxy[5- ³ H]phenyl) propenoic Acid (Caffeic Acid)	0.38%	0.11%	—
3-(4-Hydroxy-[3- ³ H]phenyl) propenoic Acid (<u>p</u> -Coumaric Acid)	0.97%	0.04%	—

The natural capsaicin mixture obtained from these feedings was degraded, as previously described, by exchange, to give inactive natural capsaicin mixture. The purity of the natural capsaicin mixture was also checked by conversion into the O-methyl derivative.

The incorporation of DL-phenylalanine into natural capsaicin mixture was appreciably higher (ca 30X) than the incorporation of L-tyrosine; a repeat of this experiment gave a similar result (Table 5). The higher incorporation of L-tyrosine than DL-phenylalanine into the total phenol fraction indicates that L-tyrosine is metabolised by the plant. These results therefore suggest that DL-phenylalanine is the major precursor and L-tyrosine is only on a minor pathway. The incorporation of phenylalanine into a C_6-C_1 unit can proceed (see above) via cinnamic acids. This is supported by our feeding results (Table 5). The incorporations of the cinnamic acids were generally lower than for phenylalanine, possibly because of their lower solubility and hence poorer adsorption by the plant. The best precursor of this class was caffeic acid, the member most soluble in water. The immediate precursor of natural capsaicin mixture could be either vanillylamine or vanillin. $[5-^3H]$ Vanillylamine hydrochloride was fed to Capsicum annuum and an incorporation was observed. To gain an insight into the fate of the $-NH_2$ group of vanillylamine a sample labelled at the benzylic position with tritium and at the 5-position with tritium was fed to Capsicum annuum. Again, an incorporation was

observed, but degradation of the capsaicin has not yet been completed.

It was of interest to see if phenylalanine was incorporated into the two main fractions of natural capsaicin mixture. A portion of the natural capsaicin mixture, derived from phenylalanine, was diluted with inactive material and methylated. The O-methyl derivative was separated into O-methyl capsaicin olefin fraction and O-methyl capsaicin dihydro fraction, by thick layer chromatography. The O-methyl capsaicin olefin fraction had an activity of 10.9 c./mg./sec., and the O-methyl capsaicin dihydro fraction an activity of 2.22 c./mg./sec. (from O-methyl natural capsaicin mixture, 4.7 c./mg./sec.). These figures correspond to there being 28% of the dihydro fraction in natural capsaicin mixture. The specific activity of the dihydro fraction was ca 5 times that of the olefin fraction, and indeed, the incorporation into the dihydro fraction was greater than the incorporation in the olefin fraction, even though the dihydro fraction is the minor component (28%). The high specific activity of the dihydro fraction is consistent with dihydrocapsaicin being a precursor of capsaicin. Conversely, it is unlikely that capsaicin could be a precursor of dihydrocapsaicin. However, they may not be interconnected and the pathway to the two compounds may diverge at an earlier point. These points could be settled by a series of feedings, the plants being worked up at various times and the activities of the two fractions being determined. An obvious test for interconversion is

to feed the labelled dihydro-and olefin-fractions separately.

A start has been made on the biosynthesis of the side chain, but so far no definite conclusions have been reached. The incorporation of sodium [2-¹⁴C] acetate was disappointingly small (0.002%). Degradation of the capsaicin has been deferred until more material is

DL-[2-¹⁴C]Mevalonic lactone was fed to Capsicum annuum and the crude coloured capsaicin-fraction was active indicating that this precursor was adsorbed and metabolised. However, repeated crystallisation, with charcoaling, removed the pigments and essentially inactive natural capsaicin mixture was obtained. This negative result suggests that the terminal group of the side chain is not derived from an isoprene unit. The biosynthesis of the rest of the side chain requires more work and the interpretation could prove to be difficult because of the presence of homologues.

Experimental

General

Melting points were taken on a Kofler block and are uncorrected. N.m.r. spectra were recorded by Mrs. I. Boston on a Varian A-60 spectrometer on permanent loan to Professor D.H.R. Barton or by Mr. P.N. Jenkins on a Varian HA100 spectrometer. Mass spectra were recorded by Mr. P. Boshoff on an A.E.I. M.S.9. spectrometer. Ultraviolet and infrared spectra were recorded on Unicam SP 800 and SP 200 spectrophotometers respectively. Microanalyses were carried out by the staff of the microanalytical laboratory.

Vanillin Oxime

Vanillin (10 g.) was dissolved in a solution of sodium hydroxide (3.3 g.) in hot water (40 ml.). A solution of hydroxylamine hydrochloride (5.8 g.), in hot water (14.6 ml.) was added, and the whole shaken until crystals formed. The solution was left overnight in the refrigerator. The crystals formed were filtered off and crystallised from water to give white platelets, m.p. 120-121.5°. Yield 9.5 g. (86.5%). $\nu_{\text{max.}}$ 1635, 3550 cm^{-1} (nujol mull). (Lit.⁽⁵⁹⁾, m.p. 121°).

Vanillylamine Hydrochloride

Vanillin oxime (1 g.), in ethanol (40 ml.), with three equivalents of hydrochloric acid, was hydrogenated at atmospheric pressure using 10% palladised charcoal as a catalyst. The solution was filtered through celite, evaporated to dryness, and the residue crystallised from ethanol/ether. Yield 1 g., (88%), m.p. 224-227°. (Lit.⁽⁶⁰⁾, m.p. 227°).

Decanoyl Chloride

n-Decanoic acid (6 g.) and purified thionyl chloride (6 ml.) were refluxed together until no more hydrogen chloride was evolved. The solution was evaporated on the water pump and the residue distilled at reduced pressure. Yield 5.12 g., b.p. 114-116° (9 mm. Hg.),

n_D^{23} 1.4383. $\nu_{\max}$ 1780 cm^{-1} (liquid film), (Lit.⁽⁶¹⁾),
b.p. 151°/18 mm. Hg.).

N-Vanillyldecanamide

Vanillylamine hydrochloride (1.5 g.), triethylamine (1.56 ml.), and n-decanoyl chloride (1.5 g.) in dry ether (100 ml.) were refluxed together for one hour. The ethereal solution was washed successively with dilute hydrochloric acid, water, saturated sodium bicarbonate solution, and water. The ethereal solution was dried (anhydrous sodium sulphate) and evaporated. The residue was heated in 20% aqueous sodium hydroxide solution at 100° for thirty minutes. The solution was then cooled, extracted with ether, and neutralised with carbon dioxide. The precipitated amide was extracted with ether. The ether extract was dried and evaporated. The residue was crystallised from di-isopropyl ether. Yield 2 g., (81.5%), m.p. 61-2°, (Lit.⁽⁶²⁾, m.p. 59-60°).
 $\nu_{\max}$ 1645, 3500, 3600 cm^{-1} (nujol mull).

Methyl Isobutyrate

Absolute methanol (20 ml.), saturated with hydrogen chloride, and isobutyric acid (22 g.), were refluxed together for three days. The solution was poured into water and extracted with ether. The ether extract was washed with saturated sodium bicarbonate solution, dried (anhydrous sodium sulphate), and fractionally distilled at atmospheric

pressure. Yield 15.2 g. (60%), n^{20}_D 1.3826, $\nu_{\max}$ 1725 cm^{-1} (liquid film), (Lit. ⁽⁶²⁾, b.p. 91°, n^{20}_D 1.3830).

2-Methyl-1,1-diphenylpropan-1-ol

Methyl isobutyrate (5 g.) in ether (30 ml.) was added to a solution of phenyl magnesium bromide (from bromobenzene (15.4 g.), and magnesium turnings (2.6 g.) in ether (50 ml.)), in ether and the solution was refluxed for one hour. The reaction mixture was then cooled in ice and acidified with dilute sulphuric acid (5 g. of conc. in 50 ml. of water). The aqueous layer was run off, and the ether layer layer washed successively with water, saturated sodium bicarbonate solution, and water. The ethereal solution was dried (anhydrous sodium sulphate), and evaporated on the water pump. The crude alcohol was used directly in the next experiment. $\nu_{\max}$ 3550 cm^{-1} (liquid film).

2-Methyl-1,1-diphenylprop-1-ene

The crude alcohol from the previous experiment was treated with phosphorus oxychloride (3 g.) in pyridine (4 g.), at room temperature for two days. The mixture was then poured onto ice and extracted with ether. The ether extract was washed successively with dilute hydrochloric acid, water, and saturated sodium bicarbonate solution. The ether extract was dried (anhydrous sodium sulphate) and evaporated. Yield 4 g., n^{24}_D 1.5830, (overall yield 34% based on methyl

isobutyrate), (Lit.⁽⁶³⁾, $n_{D}^{23.5}$ 1.586). The infra red spectrum showed no hydroxyl group was present.

1,1-Di-(4-chlorophenyl)-2-methylpropan-1-ol

This was prepared as for 2-methyl-1,1-diphenylpropan-1-ol, using p-chlorophenyl magnesium bromide (from p-chlorobromobenzene (10 g.) and magnesium (1.3 g.)), and methyl isobutyrate (2.5 g.). The crude product was used directly in the next stage.

1,1-Di-(4-chlorophenyl)-2-methylprop-1-ene

The above alcohol was dissolved in ether (10 ml.), saturated with hydrogen chloride, and left at room temperature overnight. The ether was evaporated and the residue refluxed with pyridine (5 ml.) overnight. The pyridine was evaporated at reduced pressure, and the residue extracted with ether. The ether extract was washed successively with dilute hydrochloric acid, water, saturated sodium bicarbonate solution, and again with water. The ethereal solution was dried (anhydrous sodium sulphate) and evaporated. The residue was crystallised from aqueous methanol. Yield 1.56 g. (20% based on methyl isobutyrate) m.p. 70-71° (Lit.⁽⁶³⁾, m.p. 71-2°).

O-Methylcapsaicin

Natural capsaicin (142 mg.) in 5% aqueous sodium hydroxide

solution, was shaken with dimethyl sulphate (0.3 ml.) for thirty minutes. More sodium hydroxide solution (2.5 ml. 5%) was added. The precipitate was filtered off, washed with 5% sodium hydroxide solution, and crystallised from aqueous ethanol. Yield 120 mg. (81%), m.p. 74.5-75.5°, $\nu_{\max}$. 1635, 3500 cm^{-1} (nujol mull.). (Lit.⁽⁵⁾, m.p. 77-78°).

n-Butyltriphenylphosphonium Bromide

n-Butyl bromide (23.5 g.) and triphenyl phosphine (40.5 g.) in nitromethane (125 ml.) were refluxed overnight. The solution was cooled and the precipitate filtered off and dried in vacuo. Yield 48 g. (75%), m.p. 238-241° (Lit.⁽⁶⁴⁾, m.p. 241-3°).

2-Methylhept-3-ene

A solution of bromobenzene (13.1 ml.) in ether (20 ml.) was added slowly to a suspension of lithium (1.76 g.) in ether (60 ml.), in a stream of dry nitrogen, such that the ether gently refluxed. The solution was left stirring overnight.

The above solution of lithium phenyl was added slowly, with stirring (under dry nitrogen), to a suspension of n-butyltriphenylphosphonium bromide (50 g.) in dry ether (150 ml.). The solution was stirred for one hour after the addition. A solution of isobutyraldehyde (11.4 ml.) in ether (20 ml.) was slowly added, with

stirring, a white precipitate being formed. The solution was stirred and refluxed for two days, then filtered and fractionally distilled. The residue, after all the ether had been removed, was redistilled to constant refractive index. Yield, 5 ml., b.p. 98° , $n^{23^{\circ}}$ 1.4085. (Lit.⁽⁶⁵⁾, n 1.4076).

The product still contained traces of ether and benzene and was further purified by gas phase chromatography on a Wilkens "Autoprep model A-700", using a 30% SE 30 on Chromosorb P column at 100° with helium as a carrier gas.

N,O-Dimethylcapsaicin

Natural capsaicin (300 mg.) in dry dimethylformamide (3 ml.) was added dropwise, under nitrogen, to a suspension of sodium hydride (140 mg., 50% dispersion in oil) in dimethylformamide (5 ml.). The solution was heated at 70° until no more hydrogen was evolved. The solution was cooled, and excess methyl iodide (1 ml.) was added. The reaction mixture was refluxed for one and a half hours, then poured into water and extracted with ether. The ether extract was dried (anhydrous sodium sulphate) and evaporated. The residue was chromatographed on alumina (10 g., Grade V), eluting with benzene/ethyl acetate (7:3). The product was an oil which could not be induced to crystallise. Yield 280 mg. (85.5%).

ν_{max} . 1635 cm^{-1} (chloroform), no OH or NH present in the i.r. spectrum.

Dihydrocapsaicin

Natural capsaicin (85.35 mg.) in absolute ethanol (5 ml.) was hydrogenated, in a micro-hydrogenation apparatus, at atmospheric pressure using 10% palladised charcoal (20 mg.) as a catalyst. The solution was then filtered through celite 545 and evaporated to dryness. The residue was crystallised from carbon tetrachloride/petroleum ether (b.p. 60-80°). Yield 72 mg. (84%), m.p. 64-5°. (Lit.⁽⁹⁾, m.p. 65°). Theoretical uptake 6.776 ml., actual uptake 5.255 ml. (77.55%).

Since this low percentage uptake was obtained, a control experiment was performed on cholesterol, when slightly above the theoretical amount of hydrogen was adsorbed.

Ozonolysis of 1,1-Di-(4-chlorophenyl)-2-methylprop-1-ene

The olefin (50 mg.) in ethyl chloride (5 ml.) was cooled to 0° during the passage of ozone. The reaction was followed by withdrawing aliquots at fifteen minute intervals and taking their u.v. spectra. When the u.v. spectrum was constant, the passage of ozone was discontinued and the solvent left to evaporate at room temperature. Water (2 ml.) and zinc dust was added to the residue and the whole steam distilled. The distillate gave a positive iodoform test. The residual solution gave 4,4'-dichloro-benzophenone, m.p. 145-6° (Lit.⁽⁶³⁾, m.p. 145°).

Ozonolysis of 2-Methylhept-3-ene

2-Methylhept-3-ene (100 mg.) in ethyl chloride (5 ml.) was cooled to 0° , and ozone passed through until no more was adsorbed (starch-iodide paper). The ethyl chloride was left to evaporate at room temperature. The residue was treated with freshly precipitated silver oxide (from silver nitrate, 1 g.) in aqueous sodium hydroxide solution. The solution was then acidified and steam distilled. The distillate was extracted with ether. The ether extract was dried (anhydrous sodium sulphate) and evaporated. The residue was shown, by vapour phase chromatography on a Pye 'Argon Chromatograph' using a P.E.G. A column at 115° with argon as a carrier gas (20 ml./min.), to be isobutyric acid (retention time 8.5 min.) and n-butyric acid, (retention time 12 min.).

Ozonolysis of O-Methylcapsaicin

O-Methyl natural capsaicin (50 mg.) in ethyl chloride (3 ml.) was cooled to -20° (CO_2 s / carbon tetrachloride) during the passage of ozone for fifteen minutes. The reaction was then worked up as above. The steam distillate was titrated with 0.1 N sodium hydroxide indicating a yield of 1.7 mg. of isobutyric acid (theoretical yield 11 mg.). Vapour phase chromatography as above indicated that the products were isobutyric acid (retention time 8 min.) and iso- or active valeric acid (retention time 14 min.). The former acid predominated (90%).

Methyl Hydrogen Adipate

Method I: Adipic acid (40 g.), absolute methanol (20 ml.) and concentrated sulphuric acid (5 ml.), were heated together on the steam bath for three hours. The solution was cooled, poured into water, and extracted with ether (extract A). The ether extract (A) was extracted with aqueous sodium bicarbonate solution. The bicarbonate extract was acidified and extracted with ether (extract B). Both extracts were dried (anhydrous sodium sulphate) and evaporated. Ether extract A yielded dimethyl adipate 28 g. b.p. $74^{\circ}/0.8$ mm. Hg., $n^{25^{\circ}}$ 1.4268 (Lit.⁽⁶⁶⁾, b.p. $128/30$ mm), $\nu_{\max}$. 1730 cm.^{-1} (liquid film). Ether extract B yielded methyl hydrogen adipate 7.4 g. b.p. $116-118^{\circ}/0.5$ mm. Hg., $n^{23^{\circ}}$ 1.4390 (Lit.⁽⁶⁶⁾, b.p. $178^{\circ}/30$ mm. Hg.). $\nu_{\max}$. $1700, 1730\text{ cm.}^{-1}$ (liquid film).

Method II: Adipic acid (50 g.), absolute methanol (11.4 ml.), and dimethyl adipate (10 ml.) were refluxed together for two hours. The reaction was worked up as above. Yield 30 g., b.p. $132-4^{\circ}/1.5$ mm. Hg., $n^{23^{\circ}}$ 1.4386, (Lit.⁽⁶⁶⁾, b.p. $178^{\circ}/30$ mm. Hg.) $\nu_{\max}$. $1700, 1730\text{ cm.}^{-1}$ (liquid film).

5-Methoxycarbonylpentanoyl Chloride

Methyl hydrogen adipate (7.4 g.) in purified thionyl chloride (20 ml.) was refluxed for two hours. The excess thionyl chloride was evaporated on a water pump and the residue distilled under reduced

pressure. Yield 7.5 g. (90%), b.p. $74^{\circ}/1.5$ mm. Hg., n^{20}_{D} 1.4466.
(Lit.⁽⁶⁶⁾, b.p. $141^{\circ}/36$ mm. Hg.) $\nu_{\max}$. 1740, 1800 cm^{-1} (liquid film).

Veratraldoxime

Veratraldehyde (20 g.) was dissolved in warm 95% ethanol (40 ml.), and a warm solution of hydroxylamine hydrochloride (10.1 g.) in water (12 ml.) was added, followed by a solution of sodium hydroxide (7.2 g.) in water (12 ml.). The solution was left overnight then ice was added and the solution neutralised with carbon dioxide. The crystalline solid was filtered off and crystallised from benzene/petroleum ether (b.p. $60-80^{\circ}$). Yield 17 g. (85%), m.p. $87-90^{\circ}$, $\nu_{\max}$. 3520 cm^{-1} (nujol mull.) (Lit.⁽⁶⁷⁾, m.p. $87-90^{\circ}$).

Veratrylamine⁽⁶⁸⁾

A solution of veratraldoxime (5 g.) in ether (50 ml.) was added dropwise, with stirring, to a suspension of lithium aluminium hydride (1.5 g.) in dry tetrahydrofuran (50 ml.). The solution was refluxed for one hour and then cooled in ice while ethyl acetate was added to destroy any excess lithium aluminium hydride. Water and sodium hydroxide pellets were then added until an upper layer separated. The upper layer was dried (anhydrous sodium sulphate) and evaporated. Yield 3 g. (65%), a liquid (Lit.⁽⁶⁹⁾, b.p. $120^{\circ}/2.5$ mm. Hg.)
 $\nu_{\max}$. 3450 cm^{-1} (liquid film).

N-Veratryladipamic Acid

Veratrylamine (3 g.) and triethylamine (1.3 ml.) in dry ether (100 ml.) were stirred during the addition of a solution of 5-methoxycarbanoylpentanoyl chloride (3.5 g.) in dry ether (25 ml.). The solution was refluxed for one hour after the addition. Dilute hydrochloric acid (excess) was then added. The ether layer was separated and washed successively with dilute hydrochloric acid, water, and finally saturated sodium bicarbonate solution. The ether extract was dried (anhydrous sodium sulphate) and evaporated. The residue was refluxed overnight with 50% alcoholic 4 N aqueous sodium hydroxide solution. The solution was cooled, and extracted with ether. The aqueous layer was acidified and extracted with chloroform. The chloroform extract was dried (anhydrous sodium sulphate) and evaporated. The residue was crystallised from chloroform/petroleum ether (b.p. 60-80°). Yield 4 g. (75.5%), m.p. 112-113.5°, $\nu_{\text{max.}}$ 1640, 1710 cm^{-1} (chloroform). (Found C, 61.0; H, 7.1; N, 4.5; $\text{C}_{15}\text{H}_{21}\text{NO}_5$ requires C, 61.0; H, 7.2; N, 4.7%).

Separation of O-Methyl Natural Capsaicin into its Constituents

This separation was carried out by thick layer chromatography on silica G plates, containing silver nitrate, prepared as follows:-

A slurry of Silica G F₂₅₄ (Merck, 60 g.) with 12.5% aqueous silver nitrate solution (120 ml.) was poured onto a clean 20 x 20 cm. glass plate in a suitable mould. The plate was removed after fifteen minutes and dried overnight in a current of air. O-Methylcapsaicin (125 mg.) in chloroform (1 ml.) was placed in a thin line on the plate about 1-2 cm. from one edge. The plate was developed either with benzene/ethyl acetate (1:1) or chloroform, a total of three times, drying the plate in air between each run. The appropriate bands were then detected either by spraying with 0.5% ethanolic 2,7-dichloro(R) fluorescein and observing the bands under a u.v. lamp, or by spraying with water when opaque bands were observed. The appropriate bands were removed, mixed with celite, placed in chromatography columns and eluted with ethyl acetate. The eluates were evaporated and the residues crystallised from aqueous ethanol. The products were further purified by vacuum sublimation at $126^{\circ}/10^{-5}$ mm. Hg. The dihydro compound was the faster running of the two components (i.r. & N.M.R. spectra). m.p. of both components $75-6^{\circ}$. (Found C, 71; H, 9.6; N, 4.6; Calc. for $C_{19}H_{31}NO_3$: C, 71.0; H, 9.7; N, 4.4; Found C, 71.6; H, 9.2; N, 4.7; Calc. for $C_{19}H_{29}NO_3$: C, 71.4; H, 9.15; N, 4.4%.)

Dihydro-O-methyl 'natural' capsaicin

O-Methyl natural capsaicin (58.3 mg.) in absolute ethanol (5 ml.) was hydrogenated at atmospheric pressure using 10% palladised charcoal

as a catalyst. Uptake 3.45 ml., 79.6% of theoretical. The solution was filtered through celite, evaporated, and the residue crystallised from aqueous ethanol to give dihydro-O-methyl natural capsaicin, m.p. 75-6°.

3-Methylbut-1-yne

Bromine (15.6 ml.) was added dropwise, with stirring, to a solution of 2-methylbut-1-ene (31.6 ml.) in methylcyclohexane (90 ml.) cooled to -72°. After the addition, the solution was stirred overnight, in the dark, at -72°. This solution of the dibromide was used directly in the next stage.

Hydrated ferric nitrate (0.2 g.) was dissolved in liquid ammonia (250 ml.) and stirred for five minutes. Sodium (0.6 g.) was then added and the solution stirred for ten minutes. More sodium (17.7 g.) was then slowly added so that the solution gently refluxed, a grey precipitate of sodamide being formed. The solution of the dibromide in methylcyclohexane was then slowly added, the solution being stirred a further four hours after the addition. A saturated solution of ammonium chloride (50 g. in 250 ml. water) was slowly added followed by enough water so that the ammonia no longer refluxed. The lower layer was siphoned off and the organic layer was washed successively three times with water, dilute hydrochloric acid, and again three times with water. The organic layer was then dried (anhydrous sodium sulphate)

and fractionally distilled. Yield 10 g. (47%), b.p. 26° (760 mm.).
(Lit. ⁽⁷⁰⁾, b.p. $26-28^{\circ}$) $\nu_{\max}$. 2120 cm^{-1} (chloroform).

Attempted Alkylation of 3-Methylbut-1-yne

The alkylation of this acetylene was tried using various procedures, as follows, none of which was satisfactory.

- a) Sodamine in liquid ammonia and propyl iodide.
- b) Sodamide in liquid ammonia and propyl bromide.
- c) Sodium hydride in dimethylformamide and propyltosylate.
- d) The lithium salt in dioxane and 1-bromo-3-phenylpropane.

O-Acetylcapsaicin

Natural capsaicin (100 mg.), and anhydrous sodium acetate (20 mg.) in acetic anhydride (3 ml.) were heated at 100° for one hour. The solution was poured onto ice and extracted with chloroform. The chloroform extract was dried (anhydrous sodium sulphate), and evaporated. Yield 101 mg. (89%). The product would not crystallise.
 $\nu_{\max}$. 1770, 1725, 3,500 16,80 cm^{-1} (liquid film).

O-Benzoylcapsaicin

Natural capsaicin (100 mg.) was dissolved in 2 N. aqueous sodium hydroxide solution (2 ml.) and shaken with benzoyl chloride (0.5 ml.) for half an hour. The product was extracted with ether, the ether

extract dried (anhydrous sodium sulphate), and evaporated. The residue was crystallised from carbon tetrachloride / petroleum ether (b.p. 60-80°). Yield 100 mg. (74.5%), m.p. 73.5-75.5° (Lit.⁽⁷¹⁾, m.p. 74°), ν_{max} . 1740, 3400, 1650, cm^{-1} (nujol mull).

Oxidation of O-Methylcapsaicin

O-Methyl natural capsaicin (50 mg.) and potassium permanganate (75 mg.) in purified acetone (25 ml.) were left at room temperature for two days. The solution was made alkaline with aqueous sodium hydroxide and evaporated. The residue was acidified and steam distilled. The steam distillate yielded, as previously described, isobutyric acid together with iso-valeric acid or active valeric acid according to v.p.c.

Methyl Isovalerate

Isovaleric acid (4 g.) in dry ether (25 ml.) was cooled to 0° during the addition of an ethereal solution of diazomethane until the solution turned a pale yellow. The ether was evaporated and the residue distilled. Yield 3.5 g. (77%), b.p. 110-118° (760 mm.) n_D^{27} 1.3898, ν_{max} , 1730 cm^{-1} . (liquid film). (Lit.⁽⁷²⁾, b.p. 115.5-116°).

Hydrogenation of O-Methyl Natural Capsaicin olefin-fraction

O-Methyl natural capsaicin olefin-fraction (25.4 mg.) in ethanol (5 ml.) was hydrogenated at atmospheric pressure using 5% palladised charcoal (5 mg.) as a catalyst. Hydrogen uptake 1.955 ml., theoretical uptake 1.934 ml..

5-Phenylpenta-2,4-dienoic Acid

Piperidine (3 ml.) was added dropwise, with stirring, to a solution of cinnamaldehyde (25.2 ml.), and malonic acid (25 g.), in dry pyridine (24 ml.). The solution turned a deep orange. After the vigorous reaction had moderated, the mixture was heated overnight on a steam bath. The reaction mixture was then cooled, and acidified with dilute hydrochloric acid. The precipitate was filtered off, washed with dilute hydrochloric acid and dried in vacuo. Yield 34.5 g. (100%), m.p. 162-6° (Lit.⁽⁷³⁾, m.p. 161-5°).

5-Phenylpentanoic Acid

5-Phenylpenta-2:4-dienoic acid (20 g.) was dissolved in a solution of sodium hydroxide (34.7 g.) in water (230 ml.) and heated at 70° during the slow addition of Raney Nickel alloy (23 g.) over four hours. A further portion of Raney nickel alloy (1.2 g.) was added, and the solution kept at 90° for two hours. The solution was filtered, the precipitate being washed with hot 2% sodium hydroxide solution

(2 x 25 ml.). The combined filtrates were poured into hot concentrated hydrochloric acid (230 ml.), and the whole digested at 90° for twenty minutes. The solution was cooled and extracted with ethyl acetate. The ethyl acetate extract was dried (anhydrous sodium sulphate) and evaporated. The residue was crystallised from ethyl acetate / petroleum ether (b.p. 60-80°). Yield 15 g. m.p. 57-8°, (73%) (Lit.⁽⁷³⁾), m.p. 57-8°).

5-Phenylpentanoyl Chloride

5-Phenylpentanoic acid (10 g.) was refluxed with purified thionyl chloride (50 ml.) for two hours. The solution was evaporated on a water pump and the residue distilled under reduced pressure. Yield 9.1 g. (82%), b.p. 140-144°/16 mm. Hg. (Lit.⁽⁷³⁾, b.p. 140-4°/16 mm.), $\nu_{\max}$ 1800 cm^{-1} (liquid film).

5-Phenylpentanol

5-Phenylpentanoyl chloride (15.6 g.) in dry ether (30 ml.) was added slowly, with stirring, to a suspension of lithium aluminium hydride (2.6 g.) in dry ether (70 ml.). The solution was left overnight. The reaction mixture was then cooled while ethyl acetate was added to destroy the excess lithium aluminium hydride. Water was then added followed by dilute sulphuric acid to dissolve the precipitate. The ethereal layer was then separated, dried, and

evaporated on a water pump. The residue was saponified with 20% alcoholic potassium hydroxide solution to remove esters apparently present in the product (i.r. spectrum). The solution was extracted with chloroform. The extract was dried (anhydrous sodium sulphate) and evaporated. The residue was distilled under reduced pressure. Yield 6.5 g. (50%), b.p. $95^{\circ}/0.4$ mm. Hg. $n^{25^{\circ}}$ 1.5148, (Lit.⁽⁶¹⁾, b.p. $85^{\circ}/0.2$ mm., $n^{25^{\circ}}$ 1.5140).

5-Phenyl-1-bromopentane

5-Phenylpentanol (6.5 g.) was refluxed with 48% hydrobromic acid (12 ml.) and concentrated sulphuric acid (2.4 ml.) for four hours. The solution was poured into water (50 ml.) and extracted with ether. The ether extract was washed with saturated sodium bicarbonate solution, dried (anhydrous sodium sulphate), and evaporated on a water pump. The residue was distilled under reduced pressure. Yield 8 g. (89%), b.p. $85^{\circ}/0.5$ mm. Hg., $n^{25^{\circ}}$ 1.5310, (Lit.⁽⁶¹⁾, b.p. $78-9^{\circ}/0.3$ mm., $n^{25^{\circ}}$ 1.5313).

α -Chlorobenzaldoxime

Benzaldoxime (25 g.) in chloroform (50 ml.) was stirred at 0° during the passage of chlorine gas for five hours. The solution was evaporated to dryness and the residue crystallised from petroleum ether (b.p. $40-60^{\circ}$). Yield 18.3 g. (57%) m.p. $45-6^{\circ}$, (Lit.⁽⁷⁴⁾, m.p. 48°).

5-n-Hexyl-3-phenyl- Δ^2 -isoxazoline

A solution of triethylamine (0.7 ml.) in dry ether (50 ml.) was added, with stirring, overnight, to a solution of α -chlorobenzaldoxime (1 g.) and oct-1-ene (1.02 ml.) in dry ether (75 ml.). The solution was filtered, evaporated to dryness, and the residue crystallised from petroleum ether (b.p. 60-80°), with charcoaling. Yield 0.2 g. (10%), m.p. 52-3°, (Found C, 77.7; H, 8.9; N, 6.2; $C_{15}H_{21}NO$ requires C, 77.9; H, 9.15; N, 6.05 %).

3,5-Diphenyl- Δ^2 -isoxazoline

The procedure used was as in the previous experiment, using triethylamine (0.71 ml.), α -chlorobenzaldoxime (1 g.) and styrene (0.75 ml.). The product was crystallised from petroleum ether (b.p. 60-80°) / benzene. Yield 0.3 g. (21%), m.p. 75-6°. (Lit.⁽⁷⁵⁾, m.p. 75-6°).

Trans-3,4,5,-Triphenyl- Δ^2 -isoxazoline

The reaction was performed as previously described using trans-stilbene (1.16 g.), α -chlorobenzaldoxime (1 g.) and triethylamine (0.94 ml.). The product was crystallised from ethanol. Yield 0.35 g. (18%), m.p. 140-141°, (Lit.⁽⁷⁶⁾, m.p. 140-141°).

Cis-Stilbene

Trans-Stilbene (10 g.) in benzene (100 ml.) in a quartz flask, under nitrogen, was irradiated on a high pressure u.v. lamp for one day. The solution was evaporated. The residue was dissolved in petroleum ether (b.p. 60-80°) and chromatographed on alumina (100 g. Grade I.), eluting with petroleum ether (b.p. 60-80°) containing 10% benzene. The appropriate fractions were combined and evaporated. Yield 1.54 g. (15.4%), (a liquid). n_D^{22} 1.6198 λ_{max} 279, 224.2, 207 m μ . (Lit. (77), n_D^{20} 1.6200).

Cis-3,4,5-Triphenyl- Δ^2 -isoxazoline

Cis- Stilbene (0.58 g.) and α -chlorobenzaldoxime (0.5 g.) in ether (100 ml.) were treated with a solution of triethylamine (0.5 ml.) in ether (25 ml.) as above. Yield 0.17 g. (18%), m.p. 168-168.5°. (Found: C, 83.6; H, 5.68; $C_{21}H_{17}NO$ requires: C, 84.3; H, 5.72%).

Reaction of Benzonitrile-N-oxide with Capsaicin

The reaction was performed as previously described using triethylamine (0.025 ml.) in ether (25 ml.), natural capsaicin (50 mg.) and α -chlorobenzaldoxime (28 mg.) in ether (50 ml.). The product was chromatographed on alumina (5 g. Grade V) eluting with benzene / ethyl acetate (1:1). The only products isolated were capsaicin and diphenylfuroxane.

Similar results were obtained using O-benzoyl-capsaicin and benzene or cyclohexane as solvents.

5-Phenylpentyltriphenylphosphonium Bromide

Triphenylphosphine (2.5 g.) and 1-bromo-5-phenyl-pentane (2 g.) were heated together at 100° for three days. The product was triturated with benzene/nitromethane until it crystallised, and then recrystallised from ethanol / ethyl acetate. Yield 2.9 g. (65%), m.p. 143.5-144.5°, (Found C, 71.5; H, 6.4; Br, 15.9; $C_{29}H_{30}BrP$ requires C, 71.2; H, 6.2, Br, 16.3%).

Reaction of Benzonitrile-N-oxide with O-Benzoyl Natural Capsaicin

The reaction was performed as previously described using O-benzoyl natural capsaicin olefin-fraction (180 mg.), α -chlorobenzaldoxime (114 mg.), and triethylamine (0.11 ml.). The major reaction product was unreacted O-benzoylcapsaicin.

2-Methyl-8-phenyloct-3-ene

5-Phenylpentyltriphenylphosphonium bromide (4 g.) was added, with stirring, to a solution of potassium t-butoxide (2 g.) in dry dimethylformamide (100 ml.) in an atmosphere of dry nitrogen. The solution was stirred at room temperature for one hour. Isobutraldehyde (2.8 ml.) was then added, the orange colour of the solution being

immediately discharged. The solution was stirred overnight at room temperature and then at 90° for one hour. The reaction mixture was cooled, poured into ice cold water, and extracted with petroleum ether (b.p. $30-40^{\circ}$, 4 x 150 ml.). The extract was dried (anhydrous sodium sulphate), filtered, and evaporated. The residue was chromatographed on alumina (150 g. Grade I.) eluting with petroleum ether (b.p. $30-40^{\circ}$). The appropriate fractions were combined and evaporated. Yield 0.9 g. (58%), b.p. 262° . The product was further purified by vapour phase chromatography on a Wilkens "Autoprep model A-700" using a 5% SE 30 on Chromosorb P 60/80 column at 180° with helium as a carrier gas. (Found C, 89.1; H, 11.2; $C_{15}H_{22}$ requires C, 89.0; H, 11.0%)

Isomerisation of 2-Methyl-8-phenyloct-3-ene

2-Methyl-8-phenyloct-3-ene (100 mg.) in ether (10 ml.), containing iodine (10 mg.), was irradiated for twenty four hours, at room temperature, using a 300 watt. tungsten lamp. The ether solution was then washed with aqueous sodium thiosulphate, dried (anhydrous sodium sulphate), and evaporated on a water pump. Yield 91 mg. This was not the required trans isomer but mainly 2-methyl-8-phenyloct-2-ene.

3,4-Dibromo-2-methyl-8-phenyloctane

2-Methyl-8-phenyloct-3-ene (100 mg.) in carbon tetrachloride (5 ml.) was stirred at 0° during the addition of a solution of bromine

(80 mg.) in carbon tetrachloride (1 ml., standardised solution). The reaction was left overnight at 0°. The solution was then washed with aqueous sodium bisulphite solution, dried (anhydrous sodium sulphate), and evaporated on a water pump. Yield 150 mg. (81%) of a liquid.

Reaction of 2-Methyl-8-phenyloct-3-ene with Benzonitrile-N-oxide

The reaction was performed as previously described using α -chlorobenzaldoxime (77 mg.), 2-methyl-8-phenyloct-3-ene (100 mg.), and triethylamine (50 mg.). Only starting material and diphenyl furoxane were recovered.

Separation of O-Benzoyl Natural Capsaicin into its Components

This was performed as described for O-methyl natural capsaicin. The plates were developed six times with chloroform and the products crystallised from di-isopropyl ether, to give, from O-benzoyl natural capsaicin (250 mg.):-

O-Benzoylcapsaicin olefin-fraction 170 mg, m.p. 72-3°

O-Benzoyldihydrocapsaicin 33 mg, m.p. 78-9°

Oxidation of 2-Methyl-8-phenyloct-3-ene

The olefin (50 mg.) in acetone (30 ml.) containing potassium permanganate (75 mg.) was left at room temperature for three days.

Saturated sodium bicarbonate solution (2 ml.) was added. The solution was filtered and evaporated to remove all the acetone. The aqueous residue was acidified and steam distilled. The steam distillate was titrated with 0.1 N-sodium hydroxide solution using phenolphthalein as an indicator. Uptake 2.4 ml. equivalent to a 97% yield of isobutyric acid. The solution was evaporated, and the residue acidified (dilute sulphuric acid) and extracted with ether. The ethereal solution was dried (anhydrous sodium sulphate), and evaporated. The residue was isobutyric acid only, according to v.p.c. as previously described.

Dehydrobromination of 3,4-Dibromo-2-methyl-8-phenyloctane

Liquid ammonia (20 ml.) was distilled from sodium into a vacuum jacketed flask (~50 ml.), equipped with an overhead stirrer and iardice condenser. Hydrated ferric nitrate (10 mg.) was added and the solution stirred for ten minutes. A little sodium (~10 mg.) was then added and the solution stirred another fifteen minutes. The rest of the sodium (178 mg.) was then slowly added and the whole left stirring a further fifteen minutes after the addition. The olefin dibromide (0.563 g.) in methylcyclohexane (5 ml.) was then slowly added and the whole stirred a further seven hours after the addition. A saturated solution of ammonium chloride (0.4 g. in water 2 ml.) was then added followed by enough water so that the ammonia no longer refluxed on further addition. The solution was extracted with ether. The ether

extract was washed successively with water, dilute hydrochloric acid, saturated sodium bicarbonate solution and finally again with water. The ether extract was dried (anhydrous sodium sulphate), and evaporated to give a liquid yield 263 mg. The product was not the required acetylene but the corresponding vinyl bromide (n m r. spectrum), due to incomplete dehydrobromination.

N-Nitroso-N-veratryldecanamide

A solution of nitrosyl chloride (147.3 mg.) in acetic anhydride (0.9 ml.) was added slowly, with stirring, at 0°, to a solution of N-veratryldecanamide (307 mg.) and anhydrous potassium acetate (141 mg.) in acetic acid (2.7 ml.) and acetic anhydride (4.3 ml.). The solution was stirred a further fifteen minutes after the addition and then poured onto ice. The solution was stirred until a pale yellow solid separated, this was filtered off and crystallised from ethanol, below 50°. Yield 290 mg. (85%), m.p. 33-4°, (Found C, 65.15; H, 8.4; N, 8.0; $C_{19}H_{30}N_2O_4$ requires C, 65.0; H, 8.6; N 8.0%), $\nu_{\max}$. 1720 cm^{-1} (chloroform).

Thermal Decomposition of N-Nitroso-N-veratryldecanamide

The nitroso-amide (100 mg.) was refluxed in carbon tetrachloride (10 ml.) until all the starting material had disappeared (approx. 17 hrs.), the reaction being followed by t.l.c. on silica G plates

developed with methylene dichloride. The starting material was detected by the formation of blue spots on spraying with a solution of N,N'-diphenylbenzidine (10 mg.) in 85% sulphuric acid (10 ml.). The solution was then evaporated to give veratryl decanoate. Yield 78 mg. (85%), ν_{max} 1720 cm^{-1} (chloroform).

Veratryl Decanoate

Veratryl alcohol (2 g., from the reduction of veratraldehyde with sodium borohydride in methanol), decanoyl chloride (2.28 g.) and triethylamine (1.2 ml.) in ether (150 ml.) were refluxed for four hours. The solution was then filtered, washed with sodium bicarbonate solution, dried (anhydrous sodium sulphate), and evaporated on a water pump. The residue was purified by chromatography on alumina (150 g., Grade III), eluting with methylene dichloride. A sample was distilled at $95^{\circ}/2 \times 10^{-5}$ mm. Hg. Yield 2.23 g., (58%), (Found C, 70.9; H, 9.45; $\text{C}_{19}\text{H}_{30}\text{O}_4$ requires C, 70.8; H, 9.4%).

O-Benzyl dihydro Natural Capsaicin

Dihydro natural capsaicin (300 mg.), anhydrous potassium carbonate (0.3 g.), and benzyl chloride (126.5 mg.) in purified acetone (10 ml.) were stirred and refluxed for two days. The solution was filtered, and evaporated. The residue was dissolved in ether and extracted with aqueous sodium hydroxide solution to remove unchanged capsaicin. The

ether extract was dried (anhydrous sodium sulphate) and evaporated. The residue was crystallised from di-isopropyl ether / petroleum ether (b.p. 60-80°). Yield 200 mg., m.p. 93-4°, (Found C, 75.6; H, 9.1; N, 3.6; $C_{25}H_{35}NO_3$ requires C, 75.6; H, 8.9; N, 3.5%)

Approximately 100 mg. of unreacted capsaicin was recovered.

O-Benzyl-N-nitrosodihydro Natural Capsaicin

O-Benzylidihydro natural capsaicin (100 mg.) and anhydrous potassium acetate (40 mg.) in acetic anhydride (2.15 ml.) and acetic acid (1.35 ml.), containing a trace of phosphorus pentoxide, were stirred at 0° during the addition of a solution of nitrosyl chloride (50 mg.) in acetic anhydride (0.6 ml.). The solution was stirred a further fifteen minutes at 0° and then poured onto ice. The precipitated solid was filtered off and dried in vacuo. Yield 104.5 mg. (81%), $\nu_{\max}$. 1720 cm^{-1} (chloroform); used directly in the next stage.

Thermal Decomposition of O-Benzyl-N-nitrosodihydro Natural Capsaicin

O-Benzyl-N-nitrosodihydro natural capsaicin (104.5 mg.) in carbon tetrachloride (5 ml.) was refluxed until no more nitroso compound was detectable, as previously described. The product was purified by chromatography on silica (Bpkin and Williams, M.F.C.), eluting with

methylene chloride. The appropriate fractions were combined and evaporated. The product was further purified by molecular distillation (b.p. $120^{\circ}/10^{-5}$ mm. Hg.) to give O-benzyl-vanillyl 8-methylnonoate. Yield 55 mg. (55%).

Hydrolysis of O-Benzyl-vanillyl 8-methylnonoate

The ester (55 mg.) was treated with aqueous ethanolic potassium hydroxide solution (0.1 N, in water (3 ml.) and ethanol (7 ml.)) at room temperature for five minutes. The solution was then evaporated and the residue dissolved in water (5 ml.). The aqueous solution was extracted with ether (3 x 10 ml.). The ether extract was dried (anhydrous sodium sulphate), and evaporated to give the alcohol portion of the ester. The residual aqueous extract was then acidified with dilute (6N) sulphuric and extracted with ethyl acetate. The extract was dried (anhydrous sodium sulphate) and evaporated to give the acid portion of the ester. The alcohol portion, O-benzylvanillyl alcohol, was further purified by chromatography on a 20 x 20 cm Silica G F₂₅₄ thin layer plate developed with benzene / ethyl acetate (1:1). The appropriate region was removed and extracted with ether. The ether extract was evaporated and the residue crystallised from di-isopropyl ether. Yield 18 mg. (53%), m.p. $71-72^{\circ}$. (Lit.⁽⁷⁸⁾, m.p. $72-3^{\circ}$.)

The acid portion was similarly purified on one Silica G F₂₅₄ thin layer plate eluting with petroleum ether (b.p. 60-80°) / acetic acid (8:1). Yield 9 mg. (38%).

Counting Methods

Compounds labelled with ^{14}C were counted as thin films on planchettes using a gas flow (methane) proportional counter. Tritium activities were measured using a Liquid Scintillation counter (Isotopes Developments Ltd Type 6012A). The efficiency was optimised and measured using standard $[1,2-^3\text{H}_2]\text{n-hexadecane}$, efficiencies of 16-26% being obtained. Compounds for counting were dissolved in liquid scintillator (1.2 ml., Nuclear Enterprises Ltd type N.E. 213) where possible. If they were not soluble enough, dimethylformamide (up to 0.2 ml.) was added with a corresponding drop in counting efficiency. In several cases, notably for the substituted annamic acids, the compounds (about 1 mg.) were dissolved in dimethylformamide (5-10 ml.) and aliquots (0.1 ml.) were taken, diluted with liquid scintillator (to 1.2 ml.) and counted. All compounds were counted in duplicate, very active compounds, used as precursors, being accurately diluted before counting.

$[5-^3\text{H}]$ Vanillin ⁽⁴⁹⁾

Vanillin (304 mg.), triethylamine (0.25 ml.) and tritiated water (0.3 ml., 1 c./5 ml.) was sealed in a micro-Carius tube under nitrogen. The tube was heated in a boiling water bath at 100°C for three days. It was then opened and the contents transferred to a separating funnel, acidified with dilute (6N) sulphuric acid and extracted with

chloroform. The chloroform extract was dried (anhydrous sodium sulphate) and evaporated. The residue was purified by chromatography on alumina (15 g. Grade V) eluting with benzene/chloroform (1:1). Yield 268 mg. (88%).

[5-³H] Vanillin oxime

[5-³H] Vanillin (182 mg.) was converted into the oxime using hydroxylamine hydrochloride and sodium hydroxide as previously described. The product was crystallised from water. Yield 170 mg. (85%).

[5-³H] Vanillylamine Hydrochloride

[5-³H] Vanillin oxime (50 mg.) in water (2 ml.), dioxan (2 ml.) and 6N-hydrochloric acid (0.2 ml.) was hydrogenated at atmospheric pressure using 10% palladised charcoal (5 mg.) as a catalyst. The solution was filtered through celite and evaporated to dryness. The residue was crystallised from ethanol. Yield 33 mg. (58%). A small portion (ca. 1 mg.) was diluted with inactive material and then converted into the N-benzoyl derivative for counting. Hence the activity of the original vanillylamine hydrochloride is 132 mc./m.mole.

Protocatechuic Aldehyde

This was prepared, as described in the literature⁽⁷⁹⁾, by demethylation of vanillin using aluminium trichloride and pyridine in methylene dichloride.

p-O-Benzylprotocatechuic Aldehyde

This was prepared by benzylation of protocatechuic aldehyde as described in the literature⁽⁸⁰⁾.

N-Benzoylvanillylamine

Vanillylamine hydrochloride (200 mg.), triethylamine (0.3 ml.) and benzoyl chloride (0.128 ml.) in dry ether (10 ml.) were stirred at room temperature overnight. The solution was then filtered and evaporated. The residue was dissolved in alcoholic potassium hydroxide solution and left at room temperature for one hour. The solution was then diluted with water and extracted with chloroform. The chloroform extract was dried (anhydrous sodium sulphate) and evaporated. Yield 200 mg., m.p. 140-141° (Lit.⁽⁶⁾ m.p. 140-142°).

O-Benzylvanillin

This was prepared by benzylation of vanillin as described in the literature⁽⁸¹⁾.

4-Benzyloxy-3-methoxyphenyl- α -morpholineacetonitrile

Sodium cyanide (1.5 g.) in water (5 ml.) was added dropwise, with stirring, to a solution of O-benzylvanillin (7 g.) and morpholine perchlorate (5.9 g.) in anhydrous morpholine (30 ml.). The solution was stirred and heated at 90° for two hours. The solution was cooled and diluted with water. The solid which separated was filtered off and washed with water. The product, 4-Benzyloxy-3-methoxyphenyl- α -morpholine-acetonitrile was crystallised from di-isopropyl ether / chloroform. Yield 7.6 g. (78%), m.p. 110-111.5°, (Found C, 70.8; H, 6.5; N, 8.6. $C_{20}H_{22}N_2O_3$ requires C, 71.0; H, 6.55; N, 8.3%.)

4-Benzyloxy-3-methoxybenzaloxime

Hydroxylamine hydrochloride (0.29 g.) and sodium acetate (0.34 g.) were dissolved in the minimum of water, and ethanol (10 ml.) was added. The precipitated sodium chloride was filtered off. O-Benzylvanillin (1 g.) was added to the filtrate and the solution refluxed for one hour. The solution was diluted with water and the precipitate was filtered off, washed with water, and crystallised from aqueous ethanol. Yield 1.02 g. (96%), m.p. 112.5-114.5°. (Lit.⁽⁸²⁾, 113-5°.)

3,5-Dibromo-p-toluidine

This was prepared by bromination of p-toluidine using bromine in acetic acid as described in the literature⁽⁸³⁾.

3,5-Dibromotoluene

This was prepared by deamination of 3,5 -dibromo-p-toluidine as described in the literature⁽⁸⁴⁾.

Deuteration of 3,5-Dibromotoluene

A portion (ca 1 ml.) of a solution of 3,5-dibromotoluene (3 g.) in ether (15 ml.) was added to magnesium turnings (0.56 g.) in an atmosphere of dry nitrogen.. The formation of the Grignard derivative was initiated by gently warming the reaction. The rest of the solution was then added and the whole refluxed for four hours. The reaction mixture was cooled and deuterium oxide (0.5 ml.) was added. The solution was filtered and fractionally distilled at atmospheric pressure. Yield, 1.18 g., v p c showed the product to be two compounds, probably 3-bromo-[5-²H]toluene and [3,5-²H₂]toluene.

3-Bromotoluene

A solution of 3,5-dibromotoluene (9 g.) in ether (20 ml.) was added to activated magnesium turnings (0.87 g.), containing a crystal of iodine, at such a rate that the ether gently refluxed. The solution was stirred a further three hours and then excess water was added. The ether layer was separated, dried (anhydrous sodium sulphate) and evaporated. The residue was distilled at atmospheric pressure. Yield 5 g. (83%), b.p. 181-183°, (Lit.⁽⁸⁵⁾, b.p. 183°/760 mm.)

3-Bromo-[5-²H]toluene

This was prepared from 3,5-dibromo toluene (3 g.) as above, using deuterium oxide instead of water. Yield 1.4 g. (69%). N.m.r. showed three aryl protons (τ , 2.73, 2.95) by comparison with the methyl signal (τ 7.7).

[3-²H]Toluene

This was prepared as described above using magnesium turnings (0.28 g.), 3-bromotoluene (2 g.) and deuterium oxide (0.3 ml.). Yield 0.7 g. (65%). N.m.r. showed four aryl protons (τ , 2.9) by comparison with the methyl signal (τ , 7.67).

[3-²H]Benzylbromide⁽⁸⁶⁾

[3-²H]Toluene (0.5 g.) in carbon tetrachloride (20 ml.) was heated on a water bath at 57°, and irradiated by two 300 watt. tungsten lamps, during the addition of a solution of bromine (0.3 ml.) in carbon tetrachloride (5 ml.). The reaction mixture was left a further ten minutes after the addition, then cooled, washed with water, dried (anhydrous sodium sulphate) and fractionally distilled. Yield 0.71 g., (75%).

Diethyl α -[3-²H]Benzylacetamidomalonate

[3-²H]Benzyl bromide (0.71 g.), diethyl acetamidomalonate (1.23 g.),

and a solution of sodium ethoxide in ethanol (6.5 ml., 0.71 N), were refluxed for four hours. The solution was evaporated to dryness, and the residue extracted with chloroform. The chloroform extract was evaporated and the residue was crystallised from aqueous ethanol. Yield 230 mg., (18%), m.p. 102.5-104°. (Lit.⁽⁸⁷⁾, m.p. 104°)

β -[3-²H] Phenylalanine

Diethyl α -[3-²H] benzylacetamidomalonate (100 mg.) was refluxed in 45% aqueous **hyd**nobromic acid (1.25 ml.), under nitrogen, for eight hours. The solution was cooled, diluted with water (0.5 ml.), and evaporated to dryness. The residue was dissolved in hot water (0.5 ml.) and charcoaled. The solution was evaporated to incipient crystallisation, adjusted to pH. 6 by the addition of aqueous ammonia (6N), and was left in the refrigerator overnight. The precipitate was filtered off and dried in vacuo. Yield 40 mg. (74.5%), m.p. 214-218°, (Lit.⁽⁸⁷⁾, m.p. 219-222° (dec.)).

4-Benzoyloxy-3-methoxyphenyl- α -morpholine-[α -²H] acetonitrile

4-Benzoyloxy-3-methoxyphenyl- α -morpholine acetonitrile (2 g.) was added to a suspension of sodium hydride (0.7 g., 50% dispersion with oil) in dry dimethylformamide (75 ml.), in an atmosphere of dry nitrogen. The solution was stirred at room temperature for four hours. Deuterium oxide (0.4 ml.) was added, and carbon dioxide was passed

through the solution. The solution was then diluted with water and extracted with chloroform. The chloroform extract was dried (anhydrous sodium sulphate) and evaporated. The residue was purified by chromatography on alumina (100 g. Grade V) eluting with benzene. The product was crystallised from di-isopropyl ether. Yield 1.89 g. (94.5%), m.p. 110-111.5°. N.m.r. showed no signal for the α -proton (5.28) and hence ca 100% exchange.

Hydrolysis of 4-Benzyloxy-3-methoxyphenyl- α -morpholine- $[\alpha - {}^2\text{H}]$ acetonitrile

The morpholinonitrile (1.89 g.) in ethanol (90 ml.), water (30 ml.), and concentrated hydrochloric acid (30 ml.) was refluxed for two hours. The solution was evaporated until all the ethanol had been removed, and the aqueous residue was extracted with chloroform. The chloroform extract was dried (anhydrous sodium sulphate) and evaporated. The residue was chromatographed on alumina (100 g. Grade V), eluting with benzene to give 4-benzyloxy-3-methoxy-benz $[\text{}^2\text{H}]$ aldehyde, and with ethyl acetate to give 4-hydroxy-3-methoxy-benz $[\text{}^2\text{H}]$ aldehyde. The former compound was crystallised from di-isopropyl ether. Yield 0.6 g. (45%), m.p. 70-71°, _{max.} 2100, 2150, 1665 cm.^{-1} .

4-Benzyloxy-3-methoxyphenyl- $[\alpha - {}^2\text{H}]$ formaldoxime

4-Benzyloxy-3-methoxy-benz $[\text{}^2\text{H}]$ aldehyde (220 mg.) was converted into the oxime as previously described. Yield 222 mg. (95.5%), m.p. 113-115°.

4-Hydroxy-3-methoxyphenyl-[²H]methylamine Hydrochloride

The above oxime (180 mg.) was hydrogenated in ethanol containing three equivalents of hydrochloric acid, using 10% palladised charcoal as a catalyst. The solution was filtered through celite and evaporated to dryness. The residue was crystallised from ethanol. Yield 74 mg. (56%), m.p. 225-227° (Lit.⁽⁶⁰⁾, m.p. 227°).

4-Benzyloxy-3-methoxyphenyl- α -morpholine-[α -³H]acetonitrile

This was prepared as previously described using the morpholinonitrile (1.5 g.), sodium hydride (0.4 g., 50% dispersion with oil), and tritiated water (0.2 ml., 5 ml./c.). Yield 1.3 g. (87%).

4-Benzyloxy-3-methoxy-benz[³H]aldehyde

The corresponding morpholinonitrile (1.3 g.) was hydrolysed as previously described to give 4-benzyloxy-3-methoxybenz[³H]aldehyde (yield 0.65 g.) and 4-hydroxy-3-methoxybenz[³H]aldehyde (yield 0.120 g.). The former aldehyde was diluted and counted; activity, 1.40 mc./mmole.

4-Benzyloxy-3-methoxyphenyl-[α -³H]formaldoxime

The oxime was prepared from the corresponding aldehyde (150 mg.) using hydroxylamine hydrochloride (47 mg.) and sodium acetate (56 mg.) as previously described. Yield 155 mg., (94.5%).

4-Hydroxy-3-methoxyphenyl-[³H]methylaniline Hydrochloride

The oxime (155 mg.) was hydrogenated to the amine hydrochloride as previously described. Yield 66 mg. (60%). Counted as the N benzoyl-derivative. Activity, 1.47 mc./mmole.

[3-³H]Toluene

This was prepared as previously described using m-bromotoluene (2 g.), magnesium turnings (0.3 g.), and tritiated water (0.3 ml. 5 ml./curie). Yield, 1.1 g. (crude, 100%), used directly in the next stage.

[3-³H]Benzyl Bromide

[3-³H]Toluene (1.1 g.) was brominated as previously described. Crude yield, 1.8 g. (88%), used directly in the next stage.

Diethyl α-[3-³H]Benzylacetamidomalonate

[3-³H]Benzyl bromide (1.8 g.) was condensed with diethyl acetamidomalonate (3.12 g.), as previously described. Crude yield, 3.3 g. (100%), used directly in the next stage.

β-[3-³H]Phenylalanine

The above condensation product (3.3 g.) was hydrolysed and decarboxylated as previously described. The product, which was a

mixture of phenylalanine and glycine, was purified by ion exchange chromatography on amberlite I.R. 120(H) resin (40-120 mesh, 15 g.). Elution with 1N-hydrochloric acid gave glycine hydrochloride. Elution with 6N-hydrochloric acid gave phenylalanine hydrochloride. The [$3\text{-}^3\text{H}$]phenylalanine hydrochloride was dissolved in the minimum of water and adjusted to pH. 6 by the addition of 1N-aqueous ammonia. The solution was evaporated to incipient crystallisation and left in the refrigerator overnight. The precipitate was filtered off and dried in vacuo. Yield 120 mg. (6.5% overall, based on m-bromotoluene). Activity, 0.55 mc./m mole. (obtained by weighing out ca 1 mg. dissolving it in dry dimethylformamide (5 ml.) and diluting aliquots (0.1 ml.) with liquid scintillator (1.1 ml.) for counting.)

Monobromination of $\underline{\text{N}}$ -Vanillyldecanamide

A solution of bromine in acetic acid (1 m mole./2.3 ml.) was added to a solution of $\underline{\text{N}}$ -vanillyldecanamide (307 mg., 1 m mole.) and sodium acetate (82 mg. 1 m mole.) in acetic acid (5 ml.) and left at room temperature overnight. The solution was evaporated to dryness and the residue chromatographed on alumina (7.5 g. Grade V) eluting with benzene / ethyl acetate (70/30). The product was crystallised from di-isopropyl ether / petroleum ether (b.p. 60-80°). The m.p. rose from 69-72° to 130° on repeated crystallisation, showing that more than one compound was present.

Bromination of Capsaicin

Natural capsaicin mixture (150 mg., 0.5 m mole.) was treated with a solution of bromine and sodium acetate in acetic acid (1 m mole of each/23 ml.) and worked up as above. Yield of product, 182 mg., m.p. 84-101° (after 3 crystallisations). Again apparently not a pure compound.

Monobromination of Dihydrocapsaicin

Dihydro Natural capsaicin mixture (153.5 mg., 0.5 m mole.) was treated with a solution of bromine (0.5 m mole.) and sodium acetate (0.5 m mole.) in acetic acid, and worked up as above. The product, bromodihydro natural capsaicin mixture, was crystallised from di-isopropyl ether / petroleum ether (b.p. 60-80°). Yield, 130 mg. (67.5%), m.p. 86.5-87.5° (Found C, 56.1; H, 7.4; N, 4.0; Br, 21.45; $C_{18}H_{28}BrNO_3$ requires C, 55.95; H, 7.0; N, 3.6; Br, 20.7%).

Bromination of [5-³H]Capsaicin

Natural [5-³H] capsaicin (10 mg. 2, 466 c./mg./sec., see below for preparation) was treated with two equivalents of bromine and sodium acetate in acetic acid, as above. Yield of product 11 mg. (62%), m.p. 85-93°, activity 933.5 c./mg./sec. (31% of initial activity).

Monobromination of Dihydro[5-³H]capsaicin

Natural [5-³H] capsaicin (20 mg. 208 c./sec./mg.) was first hydrogenated to give dihydro 5-³H capsaicin (activity 181 c./sec./mg.). This was then monobrominated as before. Yield 17.6 mg. (70%), m.p. 84.5-85.5°, activity 33.7 c./mg./sec. (23.4% of initial activity). A repeat of this experiment under the same conditions gave a product with 10.8% of the initial activity.

N-(Dibromovanillyl) decanamide

N-Vanillyldecanamide (153.5 mg., 0.5 m mole.) was treated with bromine (1.1 m mole.), and sodium acetate (1.1 m mole.), as previously described. The product, N-(dibromovanillyl) decanamide, was crystallised from ethyl acetate / petroleum ether (b.p. 60-80°). Yield, 205 mg. (88%), m.p. 127-9° (Found C, 40.5; H, 5.9; N, 2.9; C₁₈H₂₇Br₂NO₃ requires C, 46.5; H, 5.85; N, 3.0%).

Dibromination of Dihydro[5-³H]capsaicin

Natural [5-³H] capsaicin (20 mg., 208 c./mg./sec) was hydrogenated and brominated as previously described. Yield of product, 11 mg. (36%), m.p. 118-127°, activity 18.8 c./mg./sec. (9% of initial activity).

N-(Dibromoveratryl)decanamide

N-(Dibromovanillyl)decanamide (200 mg.), anhydrous potassium carbonate (0.5 g.), and dimethylsulphate (0.1 ml.) in acetone (5 ml.) were stirred and refluxed overnight. The solution was filtered and evaporated. The residue was crystallised from benzene. Yield, 192 mg. (93%), m.p. 108-109° (Found, C, 47.2; H, 6.1; N, 2.75; $C_{19}H_{29}Br_2NO_3$ requires C, 47.6; H, 6.1; N, 2.9%)

Dibromodihydrocapsaicin

Dihydro natural capsaicin (144.6 mg.) was brominated as previously described using two equivalents of bromine. The product was crystallised from benzene / petroleum ether (b.p. 60-80°). Yield, 179 mg. (82%), m.p. 135-137° (Found C, 46.9; H, 6.25; N, 2.9; $C_{18}H_{27}BrNO_3$ requires C, 46.5; H, 5.85; N, 3.01%).

Vanillin acetate

This was prepared by acetylation of vanillin (10 g.) using sodium acetate and acetic anhydride. Yield, 11.1 g. (87%), m.p. 77° (Lit. ⁽⁸⁸⁾, m.p. 77°).

6-Bromovanillin

This was prepared by bromination of vanillin acetate (7.5 g.) and hydrolysis of the product. Yield 3.5 g. (39%), m.p. 178-9°, (Lit. ⁽⁸⁹⁾, m.p. 178°)

5,6-Dibromovanillin

6-Bromovanillin (3 g.) and anhydrous sodium acetate (1.1 g.) in acetic acid (200 ml.) were treated with bromine (0.71 ml.) at room temperature, an immediate precipitate being formed. The solution was kept overnight and then poured into water containing a little sodium thiosulphate to remove any excess bromine. The precipitate was filtered off, washed with water, and dried in vacuo. Yield 3.85 g. (95.5%), m.p. 217-218° (Lit.⁽⁹⁰⁾, m.p. 218°)

5,6-Dibromoveratraldehyde

5,6-Dibromovanillin (1.5 m.) in dry dimethylformamide (50 ml.) was added dropwise, with stirring, to a suspension of sodium hydride (0.3 g., 50% dispersion with oil) in dry dimethylformamide (5 ml.). The solution was stirred at room temperature for two hours. A solution of methyl iodide (0.4 ml.) in dimethylformamide (5 ml.) was added and the whole stirred a further two hours. Excess methanol was added to destroy any sodium hydride present. The reaction mixture was poured into water, and the precipitate filtered off, and crystallised from glacial acetic acid. Yield 1.1 g. (70%), m.p. 128-9° (Lit.⁽⁹¹⁾, 129-30°)

5,6-Dibromovanillin Oxime

Hydroxylamine hydrochloride (0.25 g.) and anhydrous sodium acetate (0.4 g.) were dissolved in the minimum of water (ca 1 ml.) and ethanol

(10 ml.) was added. The resulting precipitate of sodium chloride was filtered off. 5,6-Dibromoveratraldehyde (1 g.) was added to the filtrate and the whole refluxed until it had all dissolved. The solution was evaporated to dryness and the residue crystallised from aqueous ethanol. Yield 0.9 g. (86%), m.p. 191.5-192.5°, (Lit.⁽⁹⁰⁾ m.p. 187°).

Hydrogenation of 5,6-Dibromovanillin Oxime

The oxime (0.9 g.) in ethanol (50 ml.), together with three equivalents of hydrochloric acid, was hydrogenated at atmospheric pressure using 10% palladised charcoal (90 mg.) as a catalyst. The solution was filtered through celite, evaporated, and the residue crystallised from ethanol/ether. Yield of product 0.35 g.. The product was vanillylamine hydrochloride, the bromine atoms being lost in the hydrogenation (identical n.m.r. spectra.).

N-[5-³H]Vanillyldecanamide

[5-³H] Vanillylamine hydrochloride (172.7 mg.) was condensed with decanoyl chloride (348 mg.) as previously described. Yield 168 mg. (60%), activity 3,820 c./sec./mg..

Dibromination of N-[5-³H]Vanillyldecanamide

The amide (15.4 mg.) was treated with two equivalents of sodium

acetate and bromine in acetic acid, as previously described. The product was chromatographed on one 20 x 20 cm. Silica G thin layer plate, developing with chloroform/ethanol (9:1). The appropriate region was removed and eluted with chloroform. The product was crystallised from benzene / petroleum ether (b.p. 60-80°). Yield 14 mg. (60%), m.p. 121-4°, activity, 251.1 c./mg./sec. (6.5% of initial activity).

Monobromination of N-[5-³H] Vanillyldecanamide

The amide (15 mg.) was brominated using 1.2 equivalents of bromine and sodium acetate as previously described. The product was purified as above. Yield 15 mg. (79.5%), (non-crystalline), activity 2,970 c./sec./mg. (22.2% of initial activity). A repeat of this experiment under the same conditions gave a product with 26.2% of the initial activity. Repeating the experiment without sodium acetate gave a product with 50% of the initial activity.

Exchange Experiments on Natural Capsaicin

Preliminary experiments were first carried out using natural capsaicin (10 mg.), potassium t-butoxide (3.7 mg., 0.5 mole equivalents) in deuterium oxide sealed in thick-walled glass tubes. The tubes were heated at 100° for various times. They were then opened, and the contents poured into water and extracted with ether.

Thin layer chromatography of the products on silica G plates, developed with benzene / ethyl acetate (1:1), showed traces of decomposition of the capsaicin after two days. This method was therefore discontinued.

The exchange was repeated using triethylamine (1 mole. equivalent.) as a base instead of potassium t-butoxide. Heating for three days at 100° gave capsaicin with only two aromatic protons (τ 3.21) in its n.m.r. spectrum as required.

[5-³H] Capsaicin

Natural capsaicin (10 mg.), triethylamine (3.3 mg.), tritiated water (0.1 ml., 5 ml./curie.), and dimethylformamide (0.1 ml.) were sealed in a micro-Carius tube under nitrogen. The tube was opened, and the contents poured into water and extracted with ether. The ether extract was dried and evaporated. The residue was crystallised from di-isopropyl ether / petroleum ether (b.p. 30-40°). Yield 9.1 mg. (91%), activity 1.45 mc./m mole.

Exchange Experiments on N-[5-³H] Vanillyldecanamide

This was performed as previously described using triethylamine as a base, with the following results:-

Mole. proportion of triethylamine	Time (days.)	Temperature.	Exchange.
0.5	4	100°	87%
0.5	4	100°	88.5%
1.0	3	100°	67%
0.5	3	118°	97.5%

Temperatures of 100° were obtained on a boiling water bath, a temperature of 118° was obtained using refluxing acetic acid. In each case the product was crystallised three times from di-isopropyl ether / petroleum ether (b.p. 30-40°). Clearly, the last experiment gives a satisfactory result.

Exchange Experiments on Natural capsaicin

Natural capsaicin (15 mg.) was exchanged as above using 0.5 mole. equivalents of triethylamine in deuterium oxide, and dimethylformamide, at 118° (refluxing acetic acid). Yield 8.5 mg. (57%). The mass spectrum indicated 90% exchange of one proton and no dideuterated species.

Exchange Experiments on 4-Hydroxybenzaldehyde

4-Hydroxybenzaldehyde (200 mg.), and triethylamine (0.22 ml.) in deuterium oxide (0.3 ml.) were sealed in a Carius tube under nitrogen and heated at 100° for forty hours. The tube was opened, and the contents removed and evaporated to dryness. The residue was purified by chromatography on alumina (5 g., Grade V), eluting with benzene / ethyl acetate (1:1). The product was crystallised from water. Yield 120 mg. (60%). The n.m.r. spectrum indicated only 60% exchange.

4-Hydroxy[3-³H]benzaldehyde

4-Hydroxybenzaldehyde (200 mg.) was exchanged as above using tritiated water (0.3 ml., 5 ml./curie). Yield 140 mg. (70%).

3-(4-Hydroxy[3-³H]phenyl)propenoic Acid

4-Hydroxy[3-³H]benzaldehyde (100 mg.), malonic acid (188 mg.), and aniline (0.01 ml.) in dry pyridine (0.41 ml.) were stirred at room temperature overnight. The solution was heated at 50-55° for three hours, cooled, and acidified with 6N hydrochloric acid. The yellow precipitate was filtered off and crystallised from water. Yield 104 mg. (77.5%), m.p. 210-213° (Lit.⁽⁹²⁾, m.p. 210-213°), activity 2.15 mc./m mole.

Attempts at labelling *p*-coumaric acid by direct exchange failed, no *p*-coumaric acid being recovered from the exchange reaction.

acid and the yellow precipitate filtered off and crystallised from aqueous ethanol. Yield 65 mg. (50%), m.p. $203-4^{\circ}$ (Lit.⁽⁹⁴⁾), m.p. 205° (dec.)), activity 1.31 mc./m mole.

[3-³H] Benzaldehyde

[3-³H]Toluene (1.53 g.) in carbon tetrachloride (5 ml.) was added dropwise, with stirring, at 0° to a solution of chromyl chloride (3 ml.) in carbon tetrachloride (25 ml.). The solution was stirred at room temperature for three days. Water (5 ml.) was added, followed by a saturated solution of sulphur dioxide in water, until the solution turned green. The carbon tetrachloride layer was separated, dried (anhydrous sodium sulphate), and fractionally distilled. The residue (1.3 g.) was used directly in the next stage.

3-[3-³H] Phenylpropenoic Acid

[3-³H] Benzaldehyde (1.3 g.), acetic anhydride (2 g.), pyridine (1 drop) and sodium acetate (0.65 g.), were refluxed together for eight hours. The solution was poured into water and steam distilled to remove excess benzaldehyde. The residual solution was filtered through celite to remove tarry impurities. The filtrate was acidified, a white precipitate separating on cooling. The precipitate was filtered off and crystallised from water, with charcoaling. Yield, 103 mg., (overall yield 6.4%), m.p. $127.5-129.5$, (Lit.⁽⁹⁵⁾, m.p. 132.5°) activity 1.52 mc./m.mole.

[2,6-³H₂] Isovanillin⁽⁴⁹⁾

Isovanillin (304 mg.) was exchanged under the same conditions as vanillin, using triethylamine as a base. The product was purified by chromatography on alumina (15 g. Grade V) eluting with benzene/chloroform (1:1). Yield 221.8 mg. (73%), used directly in the next stage.

3-(3-Hydroxy-4-Methoxy[2,6-³H₂]phenyl)propenoic Acid

[2,6-³H₂]Isovanillin (221.8 mg.), malonic acid (222 mg.), and piperidine (1 drop) in pyridine (0.8 ml.) were heated at 100° for three hours and then refluxed for one hour. The solution was cooled, diluted with water (10 ml.), and acidified with 6N sulphuric acid. The precipitate was filtered off and crystallised from aqueous ethanol with charcoaling. Yield 137.2 mg. (53%), m.p. 232-234° (Lit.⁽⁹⁶⁾), m.p. 228°) activity 1.92 mc./mmole.

Isolation of Capsaicin from Capsaicin Oleoresin⁽⁷⁾

Capsicum oleoresin (450 g., W.J. Bush and Co. Ltd.) was dissolved in water (900 ml.) containing ammonia (25 ml., d. 0.880). The solution was stirred and heated on a steam bath until it was homogenous. Barium chloride (90 g.) was slowly added, with stirring, and the solution was left to cool. The precipitate was filtered off and dried in vacuo. The dry precipitate was continuously extracted with ether in a large soschlet for three days. The ether extract was evaporated and the residue dissolved in 0.5 N sodium hydroxide solution (200 ml.). The alkaline solution was extracted with ether until the ether extract was colourless. The aqueous extract was then neutralised with carbon dioxide and extracted with ether to give the total phenols. The extract was dried (anhydrous sodium sulphate) and evaporated. The yield at this stage varied from 12 g. to 25 g.. The total phenols (25 g.) were chromatographed on alumina (1.5 Kg., Grade V) eluting successively with benzene, benzene + 15% ethyl acetate and finally benzene + 30% ethyl acetate, collecting 200 ml. fractions. The chromatography was followed by u.v. adsorption of the eluate at 281 mμ and by thin layer chromatography on thin layer plates developed with chloroform + 5% ethanol. The appropriate fractions were combined and evaporated to give a reddish oil. Yield 6-12 g. This crude capsaicin was distilled ($120^{\circ}/10^{-5}$ mm. Hg.) and crystallised from di-isopropyl ether with charcoaling. Yield 4-6 g., m.p. $64-5^{\circ}$, (Lit.⁽⁷⁾, $64-5^{\circ}$).

Feeding of precursors

In each case, about 0.05-0.1 mc. (10-20 mg.) of the compound was dissolved in water (5 ml.), with, in the case of the substituted cinnamic acids, phenylalanine, and tyrosine, the addition of enough sodium bicarbonate to completely dissolve the compound. The resulting solutions were injected into young green pods of Capsicum annuum at the rate of 0.1 ml. per pod a day for three days. The plants were left for a week after the feedings were complete. The pods were removed from the plant, the rest of the plant being discarded, and killed by immersion in liquid nitrogen. The pods were stored in ethanol at 0° until being worked up.

Isolation of Capsaicin from Feeding Experiments

The pods were ground up in a mortar and pestle under liquid nitrogen and the product extracted continuously with acetone in a soxhlet apparatus for three days. The acetone extract was evaporated to give capsicum oleoresin. The oleoresin was dissolved in 0.5 N sodium hydroxide solution and extracted with ether. The solution was neutralised with carbon dioxide and again extracted with ether to give the total phenols. The total phenols were diluted with inactive natural capsaicin mixture (30 mg.) and purified by chromatography on alumina as above. The resulting capsaicin was crystallised from di-isopropyl ether until a constant activity had been obtained. The

activity was regarded as being constant when three consecutive crystallisations gave less than a 10% variation in activity and the O-methyl derivative had the same activity.

Feeding Experiments

[5-³H] Vanillylamine Hydrochloride

The amine hydrochloride (20 mg., 0.1 mc.) was fed to Capsicum annuum (20 pods) as above, the plant being worked up after only three days.

Pods, wet weight - 84 g.

Total phenols - 81 mg., 380 c./sec./mg. (2.9% incorporation)

Capsaicin - 30 mg., 17.1 c./sec./mg. (0.05% incorporation)

This feeding took place at the beginning of a season (May 1964).

(⁺)-[3-³H] Phenylalanine

The amino acid (12 mg., 0.033 mc.) in water (4.8 ml.) was fed to Capsicum annuum (15 pods) as before and left for seven days before isolation of the capsaicin (Summer 1965).

Pods, wet weight - 17.1 g.

Total phenols - 50 mg., 59.86 c./sec./mg. (0.93% incorporation)

Capsaicin - 30 mg. 15c./sec./mg. (0.2% incorporation)

This feeding was repeated under exactly the same conditions (Late Summer 1965, 15 mg., 0.055 mc.) with the following results:-

Pods, wet weight - 23.6 g.

Total phenols - 67 mg. 67.36 c./sec./mg., (1.19% incorporation)

Capsaicin - 30 mg. 45.15 c./sec./mg., (0.5% incorporation)

In each case the incorporation is corrected for an expected 50% loss of label in the formation of capsaicin.

4-Hydroxy-3-methoxy[5-³H]phenyl[³H]methylamine Hydrochloride

The above amine, a mixture of ring and benzylically labelled compounds (10 mg.), was fed to Capsicum annuum, as above, for seven days (Summer 1965).

Pods, wet weight - 21.2 g.

Total phenols - 76 mg. 189.2 c./sec./mg. (2.5% incorporation)

Capsaicin - 30 mg. 56.8 c./sec./mg. (0.3% incorporation)

L-[3-³H]Tyrosine

The amino acid (5 mg., 0.045 mc.) in water (5 ml.) was fed to Capsicum annuum (24 pods) as before for seven days (Summer 1965).

Pods, wet weight - 66.7 g.

Total phenols - 76 mg. 22.9 c./mg./sec. (0.53% incorporation)

Capsaicin - 30 mg. 1.05 c./mg./sec. (0.015% incorporation)

The feeding was repeated (7.5 mg., 0.07 mc. Late Summer 1965) under the same conditions with the following results:-

Pods, wet weight - 11.3 g.

Total phenols - 47 mg. 139 c./mg./sec. (1.33% incorporation)

Capsaicin - 30 mg. 1.13 c./mg./sec. (0.014% incorporation)

This incorporation is corrected for expected 50% loss of label.

3-(4-Hydroxy-3-methoxy[5-³H]phenyl)propenoic Acid

The above acid (15 mg., 0.1 mc.) was fed to Capsicum annuum, as before, for seven days (Summer 1965).

Pods, wet weight - 30.74 g.

Total phenols - 186.3 mg., 54.9 c./mg./sec. (1.47% incorporation)

Capsaicin - 30 mg. 6.04 c./mg./sec. (0.026% incorporation)

3-(3,4-Dihydroxy[5-³H]phenyl)propenoic Acid

The acid (10 mg., 0.07 mc.) in water (5 ml.), containing sufficient sodium bicarbonate to dissolve the acid, was fed to Capsicum annuum for seven days, as above (Summer 1965).

Pods, wet weight - 11.6 g.

Total phenols - 73.1 mg. 25 c./mg./sec. (0.38% incorporation)

Capsaicin - 30 mg. 17.52 c./mg./sec. (0.11% incorporation)

3-(4-Hydroxy-[3-³H]phenyl)propenoic Acid

The acid (10 mg., 0.13 mc.) was fed to Capsicum annuum as above (Summer 1965).

Pods, wet weight - 9.6 g.

Total phenols - 24.3 mg., 343.4 c./mg./sec. (0.97% incorporation)

Capsaicin - 30 mg., 5.65 c./mg./sec. (0.04% incorporation)
(corrected for 50% loss of activity).

DL-[2-¹⁴C]Mevalonic Acid Lactone

The acid (0.02 mc.) was fed to Capsicum annuum as above, for three days (July 1964).

Pods, wet weight - 75 g.

Total phenols - 221 mg. 68.8 c./mg./sec. (2.42% incorporation)

Capsaicin - 30 mg. ca 0 c./mg./sec. (0% incorporation)

Sodium [2-¹⁴C]acetate

Sodium [2-¹⁴C]acetate (4.1 mg., 0.5 mc.) was fed to Capsicum annuum, as previously described, for three days.

Pods, wet weight - 20 g.

Total phenols - 52.5 mg., 1,100 c./sec./mg. (0.06% incorporation)

Capsaicin - 105.6 mg. 3.01 c./sec./mg. (0.0017% incorporation)

Degradation of Capsaicin Obtained from Tritium Labelled precursors

The tritium labelled precursors were labelled in such a position that all the activity in the resulting capsaicin should be ortho to the phenolic hydroxyl group in capsaicin. Therefore, all the activity in the capsaicin obtained from such feeding experiments should be removed entirely by exchange with water, using triethylamine as a base, as previously described. This exchange was carried out on a small scale (7.5 mg.), in a micro-Carius tube immersed in refluxing acetic acid (118°), so that the whole of the tube was at that temperature. The product was crystallised from di-isopropyl ether.

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