STUDIES IN THE BIOSYNTHESIS OF ERYTHRINA ALKALOIDS

a thesis presented by

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This thesis is dedicated to my mother, Andrew and Diane; also to Sue, without whose interruptions, it would have been completed much sooner.

"... I'm doing the best that I can,
I admit it's getting better,
A little better all the time..."

J. Lennon & P. M^{c}Cartney.

ABSTRACT

In parallel feeding experiments, R,S-N-norprotosinomenine and tyrosine were incorporated into erythraline in Erythrina crista galli but R,S-N-norreticuline and R-N-nororientaline were not. Franck's proposed biogenesis[1] of the aromatic Erythrina alkaloids via N-norreticuline was shown to have no significance. The requirement for correct hydroxylation of 1-benzyltetrahydroisoquinoline precursors is discussed.

N-Norprotosinomenine, R,S-erysodienone, erysodine and erysopine were incorporated into α-/β-erythroidine in E. berteroana. Degradation of the isolated radioactive erythroidine showed that erysodine was specifically incorporated. R,S-N-Norreticuline and R-N-nororientaline were not precursors of the erythroidines in E. berteroana.

Various systems of introducing the precursors to Erythrina species are discussed.

An unidentified compound was absent in the seed and one month old seedling extracts but was present at a maximum in relatively high concentrations in the two weeks three days extract of E. crista galli.

CONTENTS

Review of the Literature

Contents:

A. Erythrina and related Alkaloids.

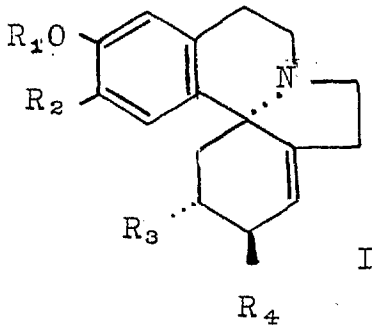
1. Isolation and Biosynthesis of the Aromatic Erythrina Alkaloids.

During recent years there has been renewed interest in the extraction of Erythrina varieties which has revealed some new naturally occurring alkaloids and some that were already known, either from synthesis or from other genera (Table 1). Reviews of the Erythrina alkaloids isolated before 1966[1] and 1968[2] have been published.

Barton[3,4,5] proposed a biogenesis (Scheme 1) for the production of the aromatic Erythrina alkaloids and by elegant labelling experiments, he has shown that the key intermediates S-(+)-N-norprotosinomenine (XIV), (±)-5,6,8,9-tetrahydro-2,12-dimethoxy-7H-dibenz [d,f] azonine-3,11-diol (XXV), and (±)-erysodienone (XVII) were incorporated into erythraline (XXXIV). Good incorporations of erythratinone (XXXI), erysotinone (XXVII), erysotine (XXVIII), erysodine (XXXV) and erysopine (XXXVI) into erythraline (XXXIV) as well as erysodine (XXXV) into erysopine (XXXVI)[6] were obtained, indicating[5] that after the basic Erythrina skeleton has been formed, alkylation and dealkylation of the phenolic groups need not necessarily follow any set order.

TABLE 1.

Erythrina Alkaloids Isolated and Identified Since 1969.

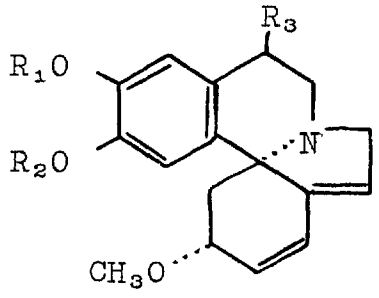


I

References

II Erythroculine R_1=CH_3, R_2=$COOCH_3$, R_3=OCH_3, R_4=H 8

III Erysotinine R_1=H, R_2=OH, R_3=OCH_3, R_4=OH 9



IV

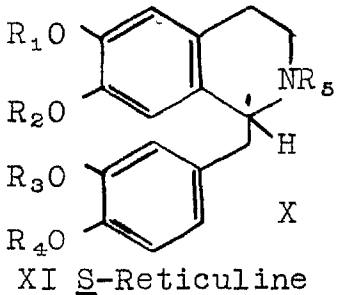
V Erythristemine IV, R_1=CH_3, R_2=CH_3, R_3=β-OCH_3 10,11,12

VI Erythrinine IV, R_1=R_2=CH_2, R_3=β-OH 13,14

VII Erysotrine IV, R_1=CH_3, R_2=CH_3, R_3=H 9,11,12,15,16,17

VIII 11-Methoxyerythraline IV, R_1=R_2=CH_2, R_3=β-OCH_3 17

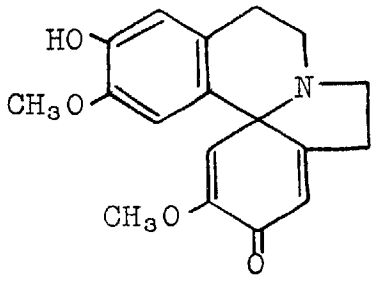
IX Erythrascine IV, R_1=CH_3, R_2=CH_3, R_3=$OCOCH_3$ 11



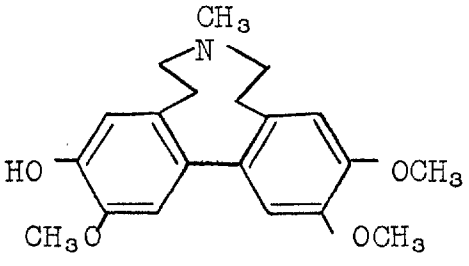
X

	R_1	R_2	R_3	R_4	R_5	C_1-H	
XI S-Reticuline	CH_3	H	H	CH_3	CH_3	α	18
XII N-Nororientaline	CH_3	H	CH_3	H	H	-	13
XIII R-N-Nororientaline	CH_3	H	CH_3	H	H	β	12
XIV S-N-Norprotosinomenine	H	CH_3	H	CH_3	H	α	16
XV Protosinomenine	H	CH_3	H	CH_3	CH_3	-	16
XVI R-Orientaline	CH_3	H	CH_3	H	CH_3	β	11,12

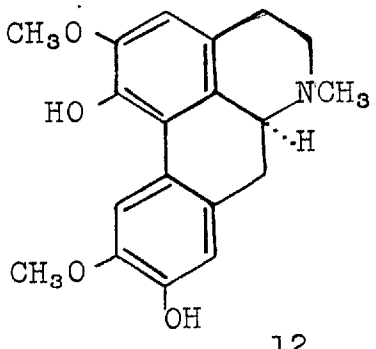
TABLE 1(cont.)



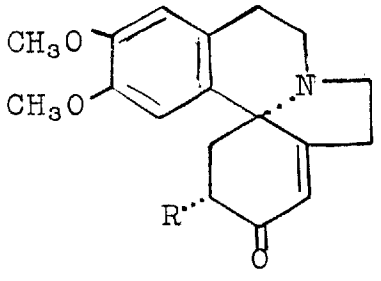
XVII Erysodienone[16]



XVIII Erybidine[14]



XIX Isoboldine[12]



XX

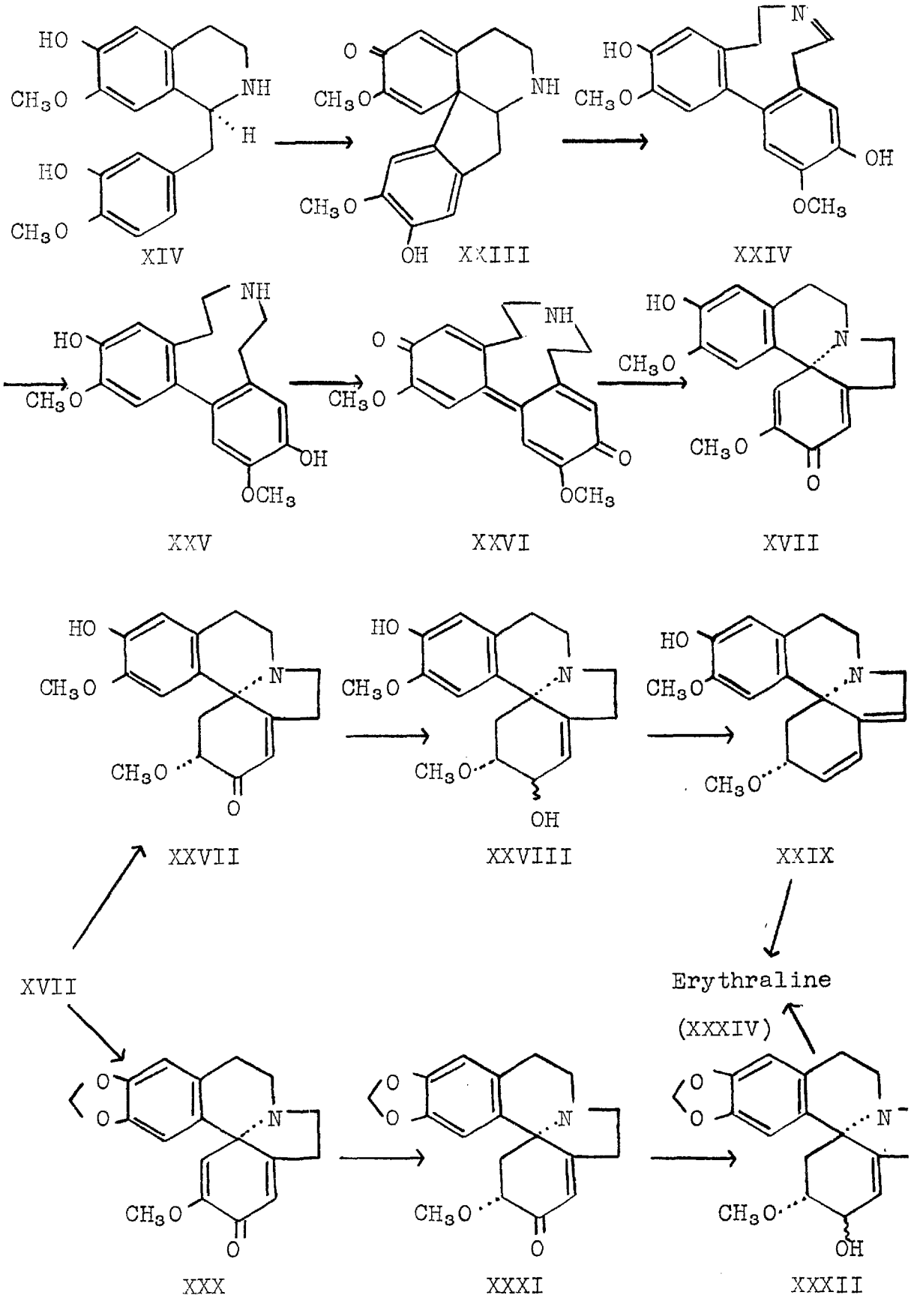
XXI Erythratidinone R=OCH_3[12]
XXII 3-Desmethoxyerythratidinone[12] R= H

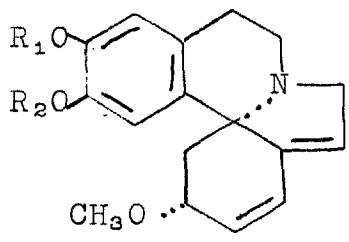
TABLE 2.

Distribution of these alkaloids throughout Erythrina and related species.

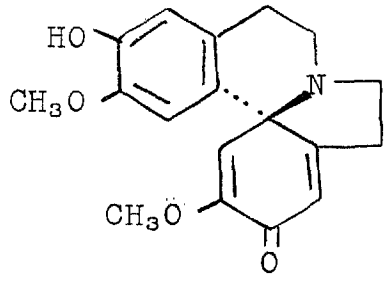
Variety	Alkaloids Identified	References
Cocculus laurifolius	II,XI	8,18
E. lithosperma	VII,XIV,XV,XVII,XXI,XXII	12,16
E. lysistemon	V,VIII	10,17
E. aborescens	V,VII,IX,XVI	11
E. poeppigiana	XIII	12
E. variegata	III,VII	9
E. indica	VII	9
E. xbidwilli lindl	VI,XII,XVIII	13,14
E. suberosa	VII	15
E. fusca	VII	12
E. abyssinica	V,XVI,XIX	12

Scheme 1


HO
CH3O
NH
H
HO
CH3O
XIV
O
CH3O
NH
CH3O
OH
XXIII
N
HO
CH3O
OH
OCH3
XXIV
NH
HO
CH3O
OH
OCH3
XXV
O
CH3O
NH
O
OCH3
XXVI
HO
N
CH3O
CH3O
O
XVII
HO
CH3O
N
CH3O
O
XXVII
HO
CH3O
N
CH3O
OH
XXVIII
HO
CH3O
N
CH3O
XXIX
Erythraline
(XXXIV)
XVII
O
O
N
CH3O
O
XXX
O
O
N
CH3O
O
XXXI
O
O
N
CH3O
OH
XXXII



XXXIII



XXXVII

XXXIV Erythraline $R_1, R_2 = CH_2$

XXXV Erysodine $R_1 = H$, $R_2 = CH_3$

XXXVI Erysopine $R_1 = R_2 = H$

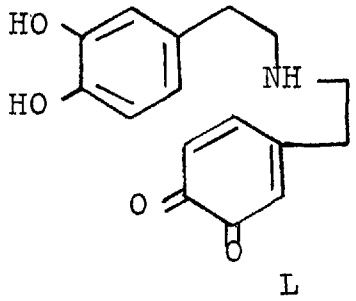
It was pointed out[5] that S-(+)-N-norprotosinomenine (XIV) would be expected to give 5R-erysodienone (XXXVII) unless a symmetric intermediate was involved since the naturally occurring aromatic Erythrina alkaloids have the 5-S configuration[3,7] (e.g. erythraline XXXIV).

The isolation (Tables 1 and 2) of S-(+)-N-norprotosinomenine (XIV), erysodienone (XVII) and erybidine (XVIII), the O- and N- dimethyl derivative of (±)-5,6,8,9-tetrahydro-2,12-dimethoxy-7H-dibenz [d,f] azonine-3,11-diol (XXV), have given further support to this scheme. Erythratidinone (XXI), the methyl ether of (XXVII, Scheme 1) and 3-desmethoxyerythratidinone (XXII) again demonstrate the ease and variety of methylation and demethylation after the Erythrina alkaloid skeleton has been produced.

The four alkaloids (V), (VI), (VIII), (IX), show the recently observed feature of oxygenation at position 11, whilst erythroculine (II) has the biogenetically unexplained presence of a C_1 unit at position 14.

2. In vitro phenol oxidations related to Erythrina alkaloid biosynthesis.

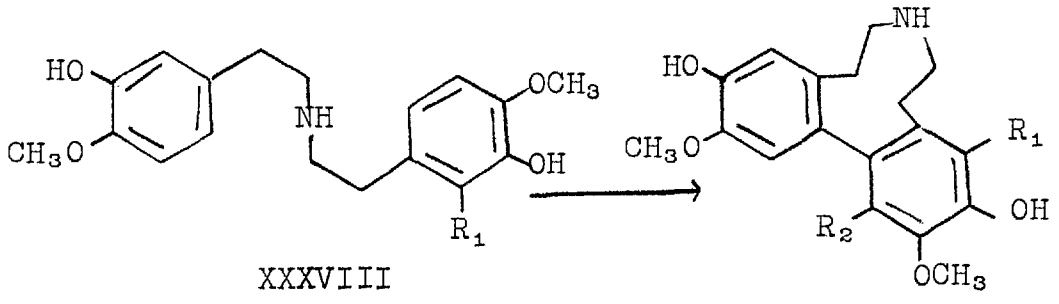
Since the concept of phenol oxidation as related to biogenesis was postulated[19,20] and amply supported experimentally, it has been a rewarding practice to attempt in vitro the proposed in vivo phenol oxidative step. Many one electron oxidising agents[21] are available for such work and from these studies some insight into the natural process may be inferred.



L

Early biogenetic theory proposed bis [2-(3-hydroxy-4-methoxyphenyl)ethyl]amine (XXXIX) as a precursor of the aromatic Erythrina alkaloids, (references cited in 3) via an o-quinone type intermediate (L). Phenol radical coupling theory[19] suggested p-p coupling of the bisphenethylamine (XXXIX) to give tetrahydrodibenz [d,f] azonine (XXV) (Scheme 2, R_1=R_2=H). In vitro support for this scheme was supplied when Mondon[22] and Scott[23] independently synthesised (±)-erysodienone (XVII) in 35% yield from bisphenethylamine (XXXIX) using alkaline potassium ferricyanide in a two phase (water/methylene chloride) system. Barton[24] has shown that the dibenzazonine (XXV) is formed in vitro by initial C-C coupling and that the mechanism for the formation of erysodienone in vitro can also be represented by Scheme 2

Scheme 2



XXXVIII

XXXIX R_1=H

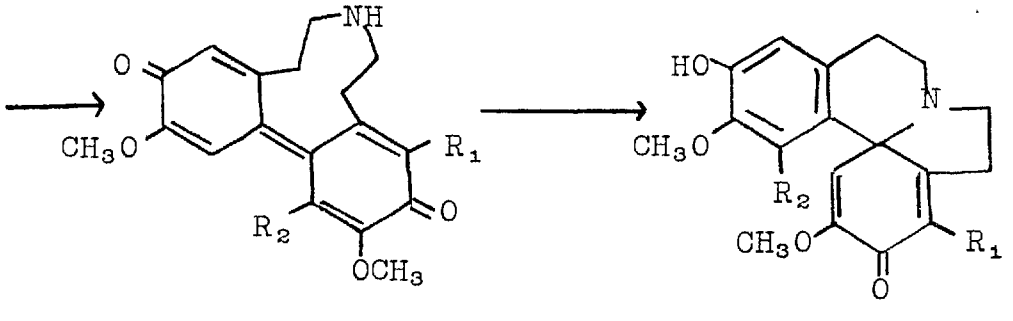
XL R_1=OCH_3

XLI

XXV R_1=R_2=H

XLII R_1=OCH_3, R_2=H

XLIII R_1=H, R_2=OCH_3



XLIV

XXVI R_1=R_2=H

XLV R_1=OCH_3, R_2=H

XLVI R_1=H, R_2=OCH_3

XLVII

XVII R_1=R_2=H

XLVIII R_1=OCH_3, R_2=H

XLIX R_1=H, R_2=OCH_3

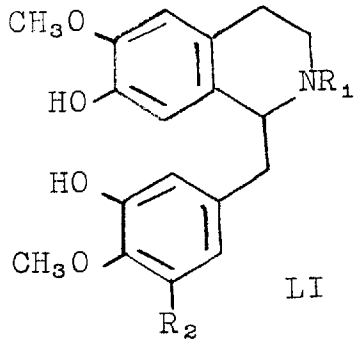
(R_1=R_2=H).

Labelled bisphenethylamine (XXXIX) was fed[3] to E. crista galli plants and gave low incorporations into erythraline (XXXIV). This led to a search for alternative precursors.

The dibenzazonine (XXV) which can be prepared by chromous ion reduction of erysodienone (XVII) was oxidised in 80% yield back to erysodienone by alkaline ferricyanide[3] via the diphenoquinone (XXVI)[24].

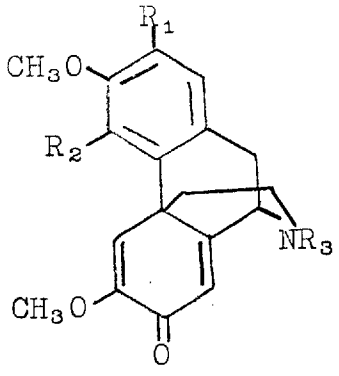
Kametani[25] has oxidised N-[2-(3-hydroxy-4-methoxyphenyl) ethyl]-2-(2,4-dimethoxy-3-hydroxyphenyl)ethylamine (XL) and obtained 1-methoxyerysodienone (XLVIII) in 5.8% yield using potassium ferricyanide, (Scheme 2, R_1=OCH_3).

Franck[26] suggested that N-norreticuline (LII) might be a biogenetic precursor for the aromatic Erythrina alkaloids when he was able to synthesise 14-methoxyerysodienone (XLIX) from N-norreticuline derivative (LIII) with an average yield of 60% per step (Scheme 3). The N-norreticuline derivative (LIII) was oxidised to the (LVIII) in 34% yield with vanadium (V) oxytrichloride. Treatment of (LVIII) with boron trifluoride produced a dienone-phenol rearrangement to give the dihydrodibenzazonine (LXII) in 63% yield. Reduction, removal of the mesyl protecting group and oxidation with potassium ferricyanide gave 14-methoxyerysodienone (XLIX) via the tetrahydrodibenzazonine (XLIII).



LI

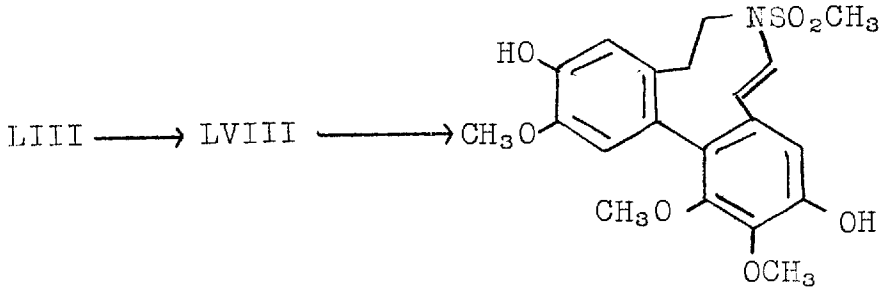
	R_1	R_2
LII	H	H
LIII	SO_2CH_3	OCH_3
LIV	COOEt	H
LV	SO_2CH_3	H
LVI	CH_3	H



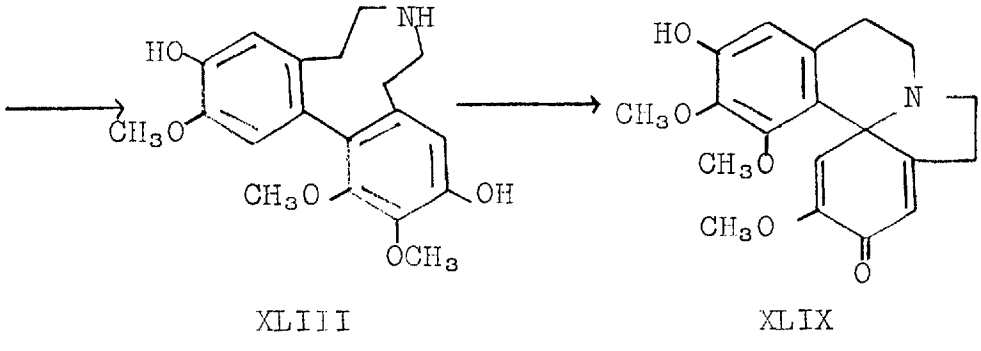
LVII

	R_1	R_2	R_3
LVIII	OH	OCH_3	SO_2CH_3
LIX	H	OH	CH_3
LX	OH	H	CH_3
LXI	OH	H	COOEt

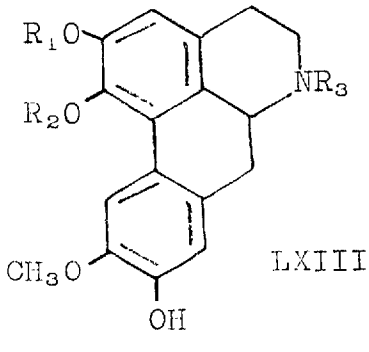
Scheme 3



LXII



XLIII XLIX



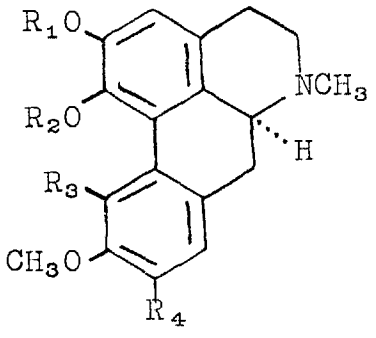
LXIII

	R_1	R_2	R_3
LXIV	CH_3	H	COOEt
LXV	CH_3	H	SO_2CH_3
LXVI	CH_3	H	CH_3
LXVII	H	CH_3	COOEt

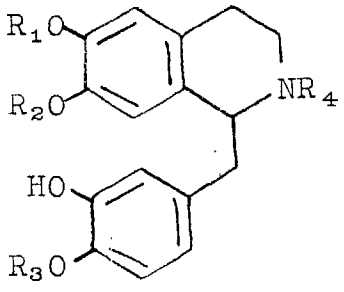
Franck[27] had earlier obtained a 40% yield for the oxidation of N-mesylnorreticuline (LV) to N-mesylisoboldine (LXV). Reticuline (LVI) can be ox- idised to isoboldine (LXVI) using potassium ferricyanide (0.5% yield)[28]; to salutaridine (LIX) using manganese dioxide (0.015% yield)[29]; to isosalutaridine (LX) (4% yield) plus isoboldine (LXVI) (6% yield) using manganese dioxide and a high dilution technique[27].

In vivo work using Dicentra eximia[30] has shown that the aporphine alkaloids corydine (LXIX), glaucine (LXX) and dicentrine (LXXI) are surprisingly derived neither via N-norreticuline (LII) nor reticuline (LVI) but via N-norprotosinomenine (LXXV). Scheme 4 has been established[30] as the pathway. The product of para-para phenol oxidative coupling (LXXVIII, R_1=H, Scheme 4) is the same as that postulated in the biosynthesis of the Erythrina alkaloids. (XXIII, Scheme 1).

An in vitro reaction analagous to the suggested in vivo para-para phenol oxidative coupling of N-norprotosinomenine has been performed by Kametani[31] (Scheme 5). N-Ethoxycarbonyl-N-norprotosinomenine (LXXXII) was oxidised using potassium ferricyanide buffered with ammonia/ammonium acetate at pH 9.2 to proerythrinadienone (LXXXVII) in 2% yield. In concentrated sulphuric acid at room temperature for one hour, the proerythrinadienone rearranged to 5,6,7a, 8-tetrahydro-3,10-dihydroxy-11-methoxy-2-oxo-dibenz[d,f]indole-7(2H)-carboxylic acid ethyl ester (XCI, Scheme 5), which



LXVIII



LXXII

LXIX $R_1=CH_3, R_2=R_4=H, R_3=OCH_3$

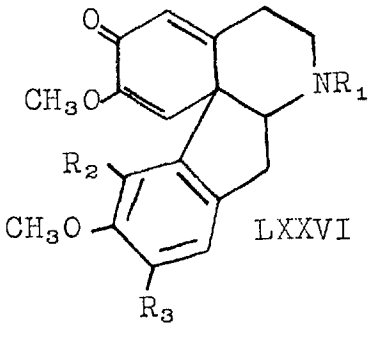
LXX $R_1=R_2=CH_3, R_3=H, R_4=OCH_3$

LXXI $R_1, R_2=CH_2, R_3=H, R_4=OCH_3$

LXXIX $R_1=R_4=H, R_2=CH_3, R_3=OH$

LXXX $R_1=R_3=H, R_2=CH_3, R_4=H$

	R_1	R_2	R_3	R_4
LXXIII	H	H	H	H
LXXIV	H	H	CH_3	H
LXXV	H	CH_3	CH_3	H



LXXVI

	R_1	R_2	R_3
LXXVII	H or CH_3	OH	H
LXXVIII	H or CH_3	H	OH

Scheme 4

LXXIII ⟶ LXXIV ⟶ LXXV ⟶ LXXVII or LXXVIII

LXXVII ⟶ LXXIX (LXVIII; $R_1=H, R_2=CH_3, R_3=OH, R_4=H$)

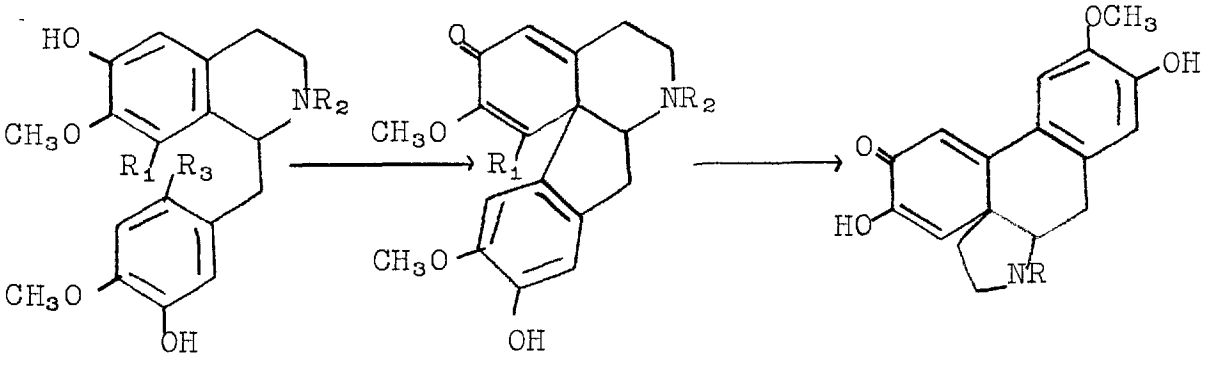
LXXIX ⟶ corydine (LXIX)

LXXVIII ⟶ LXXX (LXVIII; $R_1=H, R_2=CH_3, R_3=H, R_4=H$)

LXXX ⟶ glaucine (LXX) and dicentrine (LXXI)

was characterised by its spectral data. Two other possible rearrangement products, the aporphine (LXVII, LXIII R_1=H, R_2=CH_3, R_3=COOEt) and the morphadienone (LXI, LVII R_1=OH, R_2=H, R_3=COOEt), were discounted spectroscopically and by unambiguous synthesis respectively.

Scheme 5



LXXXI LXXXVI XC

	R_1	R_2	R_3		R_1	R_2		R
LXXXII	H	COOEt	H	LXXXVII	H	COOEt	XCI	COOEt
LXXXIII	H	COOEt	Br					
LXXXIV	OCH_3	COOEt	Br	LXXXVIII	OCH_3	COOEt		
LXXXV	H	$COCF_3$	Br	LXXXIX	H	$COCF_3$	XCII	$COCF_3$

The industrious Japanese workers have more recently improved the yield of this reaction by using a photolytic method. Kametani[32] photolysed 2'-bromo-N-ethoxycarbonyl-N-norprotosinomenine (LXXXIII) to the same proerythrinadienone (LXXXVII) as above in 12% yield. 2'-Bromo-8-methoxy-N-ethoxycarbonyl-N-norprotosinomenine (LXXXIV) photolysed in 8% yield to (LXXXVIII) and 2'-bromo-N-trifluoroacetyl-N-norprotosinomenine (LXXXV) gave (LXXXIX) in 4% yield. The N-trifluoroacetylproerythrinadienone (LXXXIX) was also treated with sulphuric acid to give N-trifluoroacetyl-dibenzindole ring system (XCII).

3. Isolation and proposed biosynthesis of the homoerythrina and Cephalotaxus alkaloids.

Cephalotaxine (XCIV) was first isolated in 1963 from Cephalotaxus drupaceae varieties harringtonia and fortunei[33]. A partial structure was deduced from n.m.r.[34] and an x-ray[35] gave the total structure and absolute configuration. The ester derivatives of cephalotaxine (Table 3), named harringtonine (XCV), isoharringtonine (XCVI), homoharringtonine (XCVII) and deoxyharringtonine (XCVIII) have been found as minor alkaloidal components in C. harringtonia. The structures of the transesterification products from the natural esters have been further investigated[36,38] but the exact position of attachment has not been published.

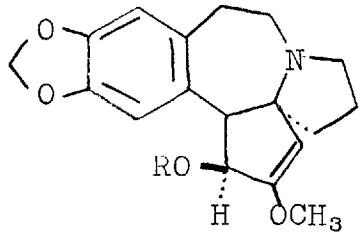
The homoerythrina alkaloids (Table 3) were isolated from Schelhammera pedunculata and were named because they have the basic Erythrina alkaloid skeleton with an additional methylene group in ring C. Schelhammerine (C), schelhammericine (CI) and schelhammeridine (CXI) were the first fully characterised alkaloids[39,40] and later seven other alkaloids[41] with the same basic skeleton (CII, CIII, CIV, CXII, CXIII, CXIV and CXVI) were reported in S. pedunculata (Lilliaceae) and S. multiflora (Tables 3 and 4).

Homoerythrina alkaloids have also been isolated from Phelline comosa[42] and Phelline billiardieri[43], (Tables 3 and 4). The alkaloids with the skeleton (CXXII) from P. billiardieri have been called homoerythioidines since the proposed δ-lactonoid ring D is analagous to that found in α- and β- erythroidine (CXXXV and CXXXVI). The structure of ring D and the stereochemistry of (CXXII) have still to be eludicated.

Table 3

Homoerythrina and Cephalotaxus alkaloids isolated before August 1972

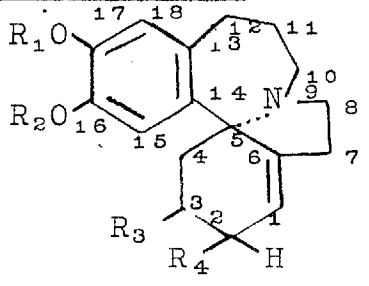
a. Cephalotaxus alkaloids



XCIII

	R	Transesterification product	References
XCIV	H		33,34,35,36,37.
XCV	$C_{10}H_{17}O_5$	$CH_3OOCCH_2C(OH)(COOCH_3)(CH_2)_2C(OH)(CH_3)_2$	36,37
XCVI	$C_{10}H_{17}O_5$	$CH_3OOCCH(OH)C(OH)(COOCH_3)(CH_2)_2CH(CH_3)_2$	36,37
XCVII	$C_{11}H_{19}O_5$	$CH_3OOCC(OH)(COOCH_3)(CH_2)_3C(OH)(CH_3)_2$	36,37
XCVIII	$C_{10}H_{17}O_4$	$CH_3OOCCH_2C(OH)(COOCH_3)(CH_2)_2CH(CH_3)_2$	37,38

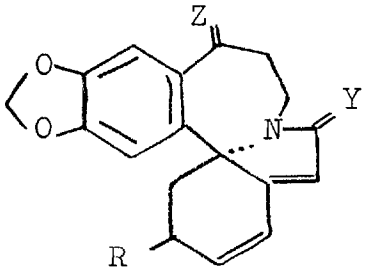
b. Homoerythrina alkaloids



XCIX

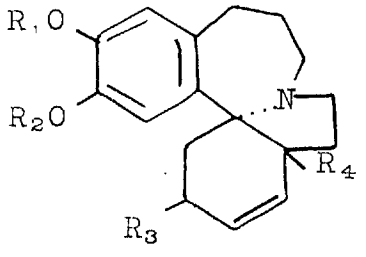
	R_1	R_2	R_3	R_4	References
C	CH_2		β-OCH_3	α-OH	39,40
CI	CH_2		β-OCH_3	H	37,39,41
CII	CH_2		α-OCH_3	H	37,41,42
CIII	CH_3	H	α-OCH_3	H	37,41
CIV	CH_3	H	α-OCH_3	α-OH	41
CV	CH_2		α-OCH_3	α-OH	42
CVI 18-methoxy	CH_3	CH_3	α-OCH_3	H	42
CVII	H,	CH_3	β-OCH_3	H	37
CVIII	CH_3	CH_3	β-OCH_3	H	37
CIX	CH_3	CH_3	α-OCH_3	H	37

Table 3 (continued)



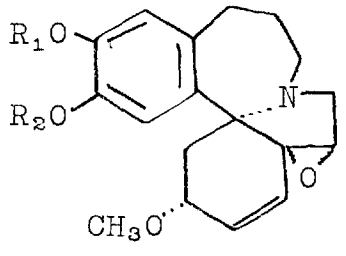
CX

	R	Z	Y	Refs.
CXI	β-OCH_3	H_2	H_2	39,40
CXII	α-OCH_3	H_2	H_2	41
CXIII	β-OCH_3	O	H_2	41
CXIV	β-OCH_3	H_2	O	41

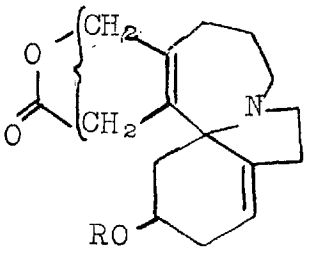


CXV

	R_1	R_2	R_3	R_4	Refs.
CXVI	CH_2		β-OCH_3	α-H	41
CXVII	CH_2		OCH_3	H	42
CXVIII	CH_3	CH_3	OCH_3	H	42



CXIX



CXXII

CXX $R_1, R_2 = CH_2$ [42]

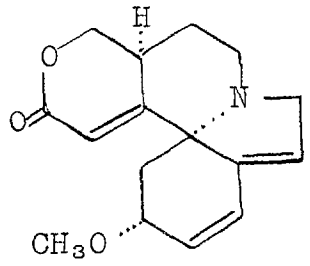
CXXI $R_1 = R_2 = CH_3$ [42]

CXXIIIa R=CH_3 [43]

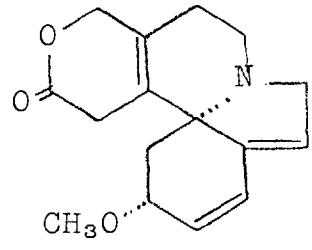
CXXIIIb R=H [43]

Table 4

Species	Structures	Refs.
C. harringtonia	XCIV, XCV, XCVI, XCVII, XCVIII, CI, CII, CIII, CVII, CVIII, CIX.	33,37
C. fortunei	XCIV	33
S. pedunculata and S. multiflora	C, CI, CII, CIII, CIV, CXI, CXII, CXIII, CXIV, CXVI.	39, 40,41.
P. comosa	CII, CV, CVI, CXVII, CXVIII, CXX, CXXI.	42
P. billiardieri	CXXIIIa, CXXIIb.	43



CXXXV



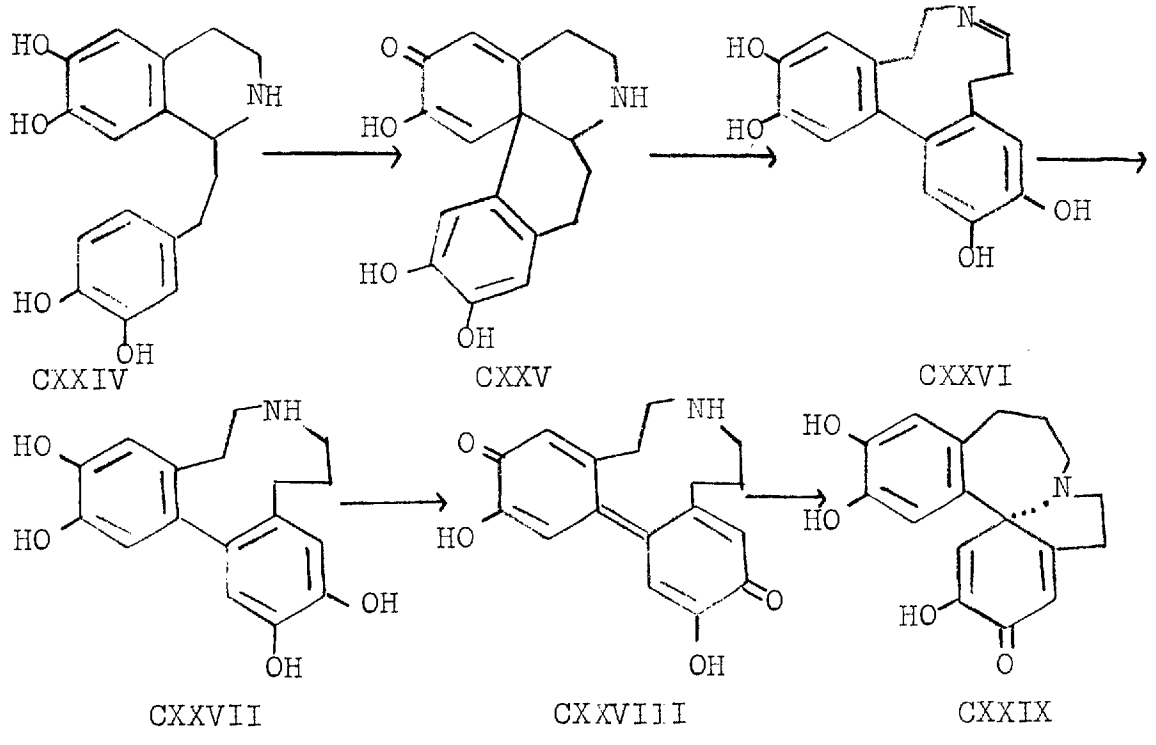
CXXXVI

From a biosynthetic and chemo-taxanomical point of view, it is interesting to find that homoerythrina alkaloids have been found in Cephalotaxus harringtonia.[37] (Table 4).

The structures deduced for the homoerythrina alkaloids (Table 3) produce a number of interesting points and some analogies to the Erythrina alkaloids. The stereochemistry at C_5 is the same in both series but in the Erythrina group only cis-(α)-C_3-methoxy or hydroxy is observed whereas in the homoerythrinas, both epimers have been identified. Generally the oxygenation pattern is the same for both series but 18-methoxy, (CVI), 8-oxo (CXIV) and 6,7-epoxy (CXIX) derivatives of the homoerythrina skeleton are exceptions. Another outstanding analogy is the presumed cleavage of the aromatic ring D of the homoerythrina alkaloids to give the homoerythroidine structure (CXXII) which has not been fully characterised.

Biogenetic speculation has produced two pathways that utilise common early intermediates and which may account for the formation of the homoerythrina and cephalotaxine skeletons. Fitzgerald[40] proposed a biogenesis (Scheme 6) of the homoerythrina series using Barton's proven scheme for the Erythrina alkaloids as a basis. Powell[37], after observing that homoerythrina alkaloids are found in Cephalotaxus species, suggested that alternative ring

Scheme 6



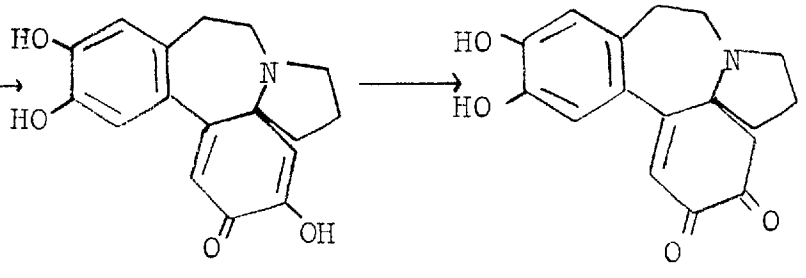
CXXIV CXXV CXXVI

CXXVII CXXVIII CXXIX

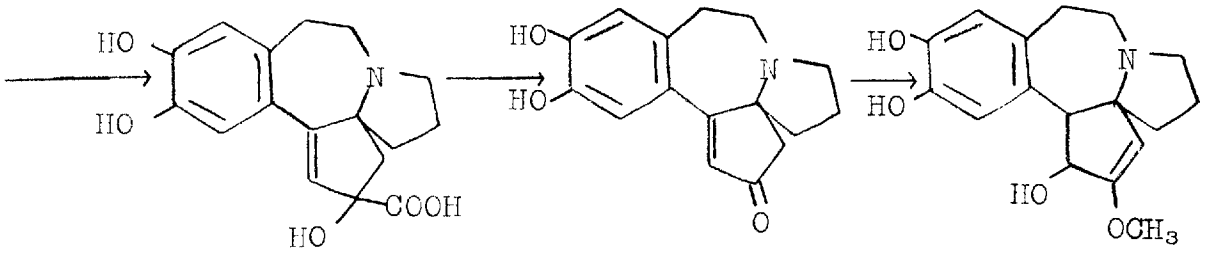
⟶ Homoerythrina alkaloids (c.f. Scheme 1)

Scheme 7

CXXVIII ⟶ (Scheme 6)



CXXX CXXXI

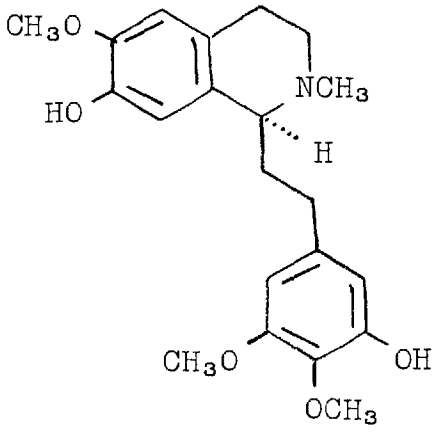


CXXXII CXXXIII CXXXIV

⟶ Cephalotaxus alkaloids

closure of (CXXVIII) followed by appropriate electron shifts, a benzilic acid type rearrangement and decarboxylation would produce the Cephalotaxus alkaloids (Scheme 7). Both schemes did not consider that if they are to be analagous to the benzyl- and phenethyl- tetrahydroisoquinoline examples, the correct methylation pattern is required[30,44,45,46] in order to direct the phenol oxidation in the desired manner.

A phenethyltetrahydroisoquinoline, (-)-autumnaline (CXXXVII) has been isolated from Colchicum cornigeum.[47]



CXXXVII

B. Cleavage of 1,2-Dihydroxy Aromatic Compounds.

1. Introduction

The need to understand how aromatic compounds are biodegraded has prompted a great deal of research into the metabolism of these compounds in micro-organisms. Many such compounds have been thrust upon Nature by Man in the form of detergents, weedkillers or industrial waste. Most detergents are alkyl benzenesulphonates and some chlorobenzene derivatives have been used as effective weedkillers. Natural oil deposits contain benzene and the polyaromatics which have been isolated and also synthesised. Such compounds can be toxic to higher living organisms, but some micro-organisms are able to biodegrade them to simple aliphatic compounds which can be accepted into primary metabolic pathways.

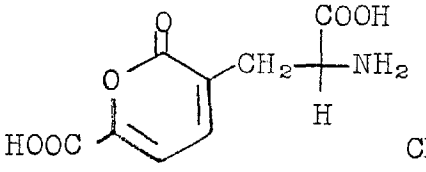
In the biodegradation of an aromatic ring, a single hydroxyl group is essential and frequently there is a requirement for a 1,2-dihydroxy system to be present[48,49,50]. It is the oxidative cleavage of the aromatic ring between (intradiol) and adjacent (extradiol) to the 1,2-dihydroxy groups and subsequent degradation which are covered by this review.

2. Aromatic Ring Cleavage in Plants.

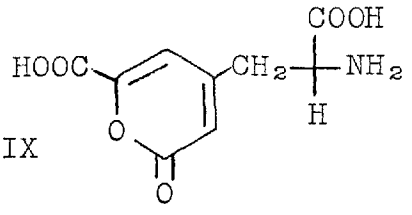
In plants there are only four known cases where cleavage of an aromatic ring has been proposed in the formation of a secondary metabolite. They are a) the alkaloids of the Stizolobium species[51,52] (stizolobinic acid (CXXXVIII) and stizolobic acid (CXXXIX); b) the betalains[53], the red-violet and yellow alkaloids of the Order Centrosperma

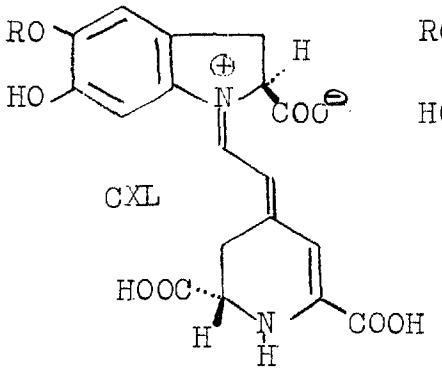
(e.g. betanin (CXLI), isobetanin (CXLIV) and indicaxanthin (CXLVI)).

CXXXVIII

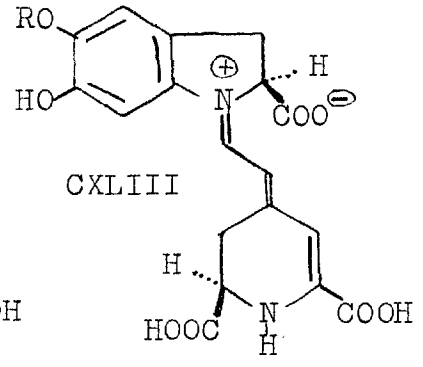


CXXXIX

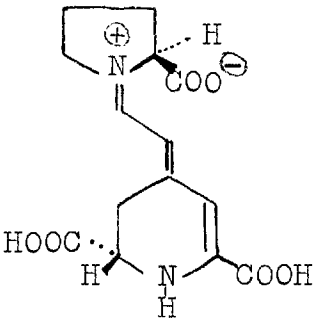




CXL



CXLIII



CXLVI

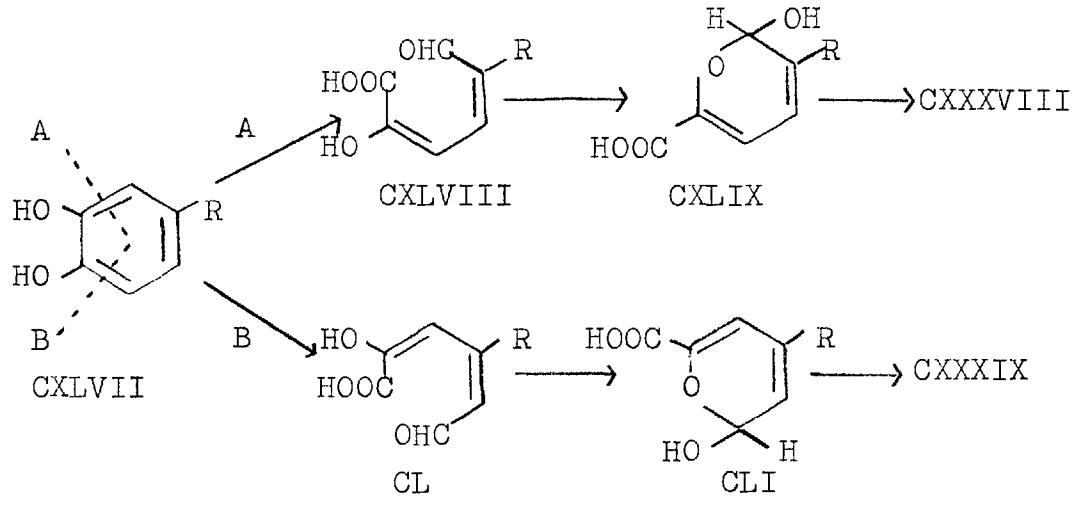
CXLI R=β-D-glucosyl
CXLII R=H

CXLIV R=β-D-glucosyl
CXLV R=H

c) the homoerythroidines[43] (CXXII Table 3) isolated from Phelline billiardieri and d) the erythroidines[54,55] (α- and β-erythroidine (CXXXV and CXXXVI) which have been isolated from Erythrina americana, E. berteroana, E. costaricensis, E. tholboniana[1], and E. poeppigiana[12].

S- Stizolobinic (CXXXVIII) and S- stizolobic (CXXXIX) acids have been isolated from Stizolobium deerianum, S. pruriens, S. utilis, Mucana irukanda[51] and S. hassjoo[51,52]. After these acids were isolated from etiolated seedlings of S. hassjoo, Senoh characterised them[52] as α- pyrone-6-carboxylic acid derivatives of S-alanine. Furthermore he postulated[48,50,52] that, in vivo, these amino acids were derived from 3-(3,4-dihydroxyphenyl) alanine (CXLVII, R=3-S-alanyl) by the two possible modes of extradiol oxidative ring cleavage (Scheme 8).

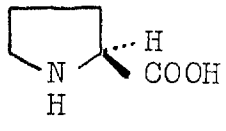
Scheme 8 (R=S-alanyl)



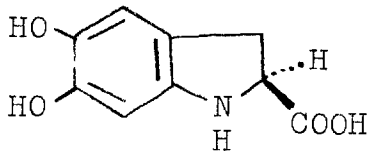
As evidence for this proposed mechanism, Senoh isolated and crystallised the enzyme, 3,4-dihydroxyphenylacetic acid 2,3-dioxygenase, from 4-hydroxyphenylacetic acid adapted cells of Pseudomonas ovalis. This enzyme[50,56,57] catalyses the formation of 5-carboxymethyl-2-hydroxy-6-oxo-hexa-2,4-dienoic acid (CXLVIII, R=CH_2 COOH) by oxidative ring fission of 3,4-dihydroxyphenylacetic acid (CXLVII, R=CH_2 COOH) between the 2',3' positions (extradiol proximal cleavage). 3-(3,4-Dihydroxyphenyl) alanine was also cleaved specifically between the 2', 3' positions[50,56,57] by this enzyme indicating that the suggested pathway (Scheme 8) for the formation of the Stizolobium acids is feasible. As far as is known, radiolabelled precursors have not been fed to the whole plant.

The red-violet and yellow alkaloids, the betalains, which occur abundantly and widely in plants (e.g. beetroot, cacti and pokeberry) and belong to ten families of the Order Centrosperma[53], have been studied since the 1860's. A crystalline sample of betanin (CXLI) was not obtained until

1957 when the complete structures were relatively quickly elucidated. The 'betalains' is an expression used to describe the class of coloured alkaloids to which the red-violet betacyanins (e.g. CXL and CXLIII) and the yellow betaxanthins (e.g. CXLVI) belong. The betaxanthins are structurally related to the betacyanins, possessing the same tetrahydropyridine unit, such that betanin (CXLI) can be converted in the presence of excess S-proline (CLII) to indicaxanthin (CXLVI). Betanidin(CXLII) is formed when indicaxanthin is reacted with excess 2-S-carboxy-5,6-dihydroxy-2,3-dihydroindole (S-cyclo D.O.P.A.) (CLIII).



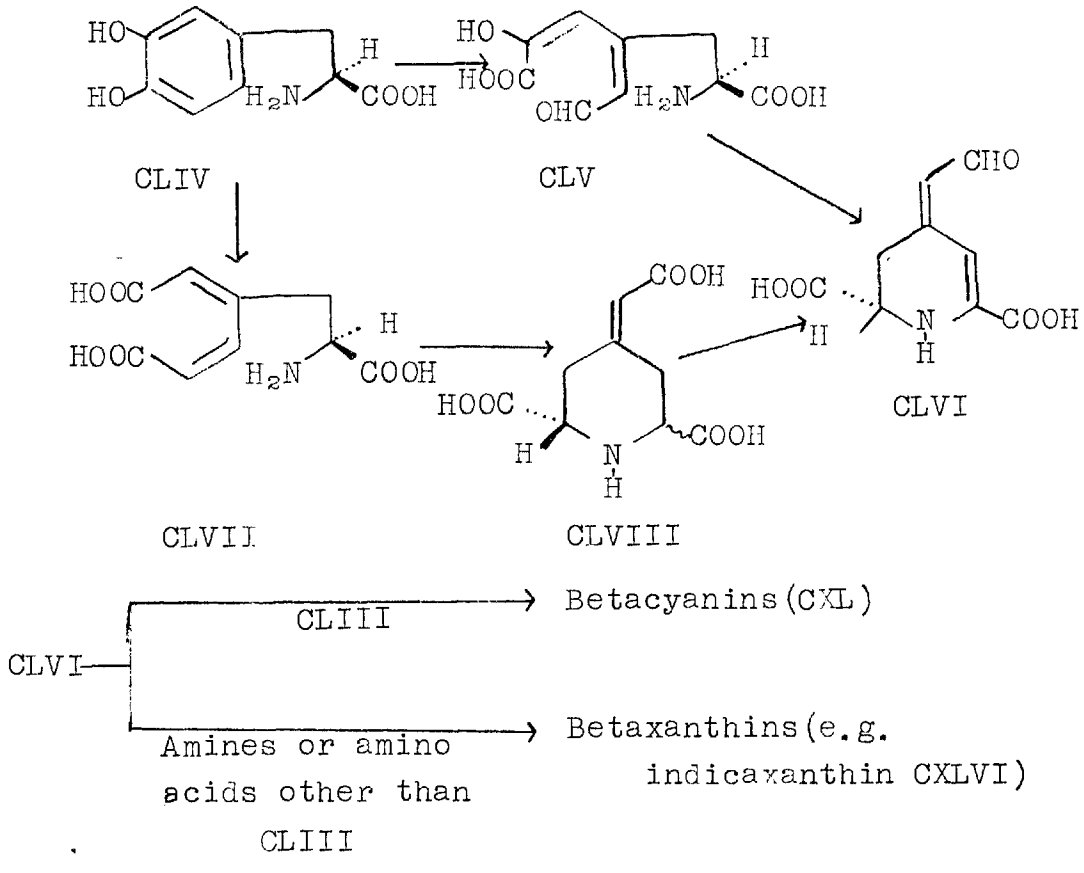
CLII



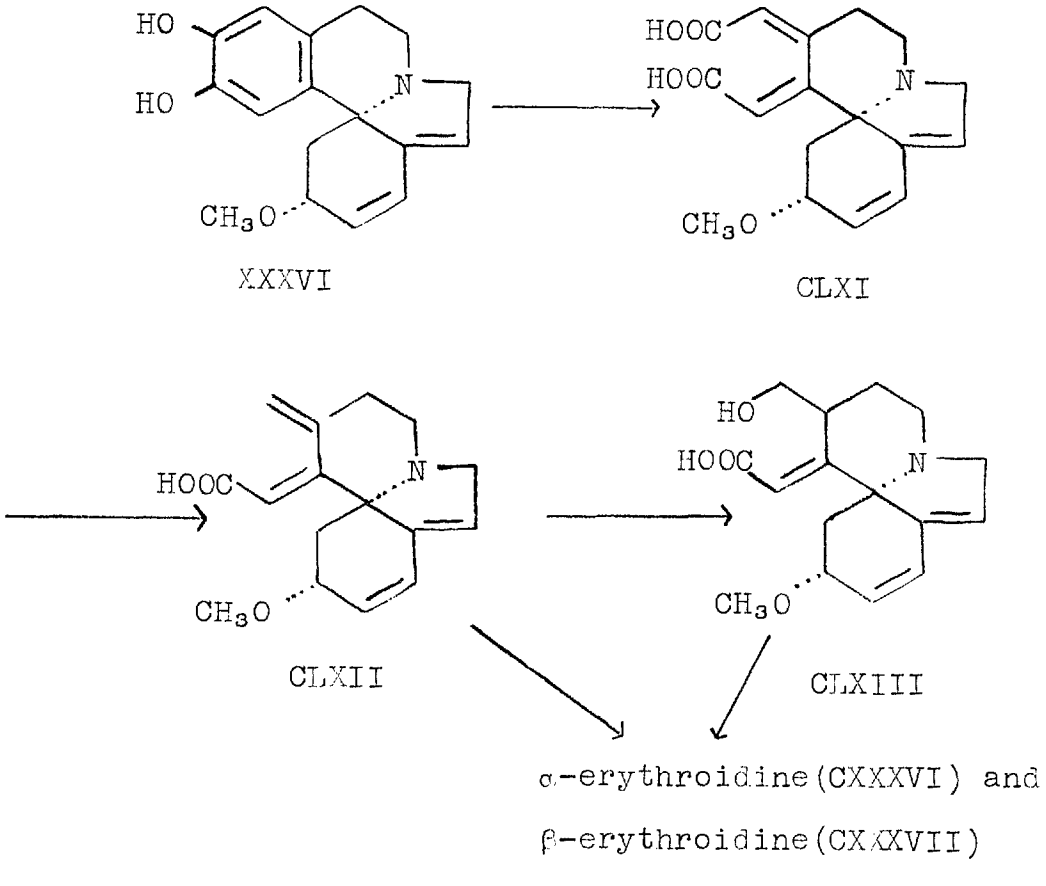
CLIII

In the proposed biogenesis of the betalains (Scheme 9),[53,60] S-3-(3,4-dihydroxyphenyl) alanine (CLIV) can be cleaved in the intradiol or extradiol positions to give the common intermediate, betalamic acid (CLVI). Condensation of betalamic acid (CLVI) with S-cyclo D.O.P.A. (CLIII) would give the betacyanins and reaction with amines or other amino acids would yield the betaxanthins. Dreiding and Mabry[60] have shown that R,S-3-(3,4-dihydroxyphenyl)-[1-^{14}C]-alanine and R,S-3-(3,4-dihydroxyphenyl-[2-^{14}C]-alanine were specifically incorporated in high yield (about 5% but greater than 10% in some experiments) into the tetrahydropyridine unit of betanin (CXLI) in cactus fruits. (Opuntia decumbens and O. bergeriana). Surprisingly neither precursor had comparable

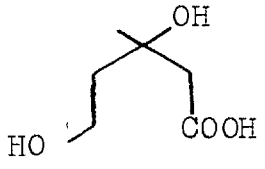
Scheme 9



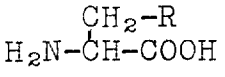
Scheme 10



incorporation (less than 1%) into the dihydroindole part of betanin whilst mevalonic acid (CLIX), aspartic acid (CLX, R=COOH) and phenylalanine (CLX, R= phenyl) had zero incorporation. These data do not distinguish between the two possible positions of cleavage (Scheme 9).



CLIX

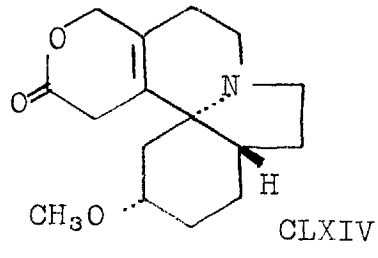


CLX

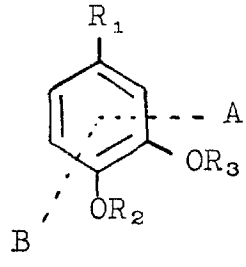
The homoerythroidine alkaloids[43] (CXXII, Table 3) have not been fully characterised. The exact constitution of the lactone ring D and the stereochemistry at positions 3 and 5 have to be determined. Hence speculation of an analagous pathway to that proposed for the erythroidines (CXXXV and CXXXVI) would not be valid.

As soon as a structure of β-erythroidine (CXXXVI) was suggested, a rational biosynthetic route to its formation was outlined.[61] The aromatic Erythrina alkaloids were thought at that time to be derived from bisphenethylamine (XXXIX) but the proposed pathways[61,62] (Scheme 10) from erysopine (XXXVI) to β-erythroidine (CXXXVI) must still be considered as possibilities. 'Woodward fission'[62,63], was postulated as the method of aromatic ring cleavage in each case. Leete[64] fed R,S-[2-^{14}C]-tyrosine (CLX, R=4-hydroxyphenyl) to 6 month old E. berteroana plants and obtained 0.025% incorporation into a mixture of α- and β-erythroidines. By degradation of tetrahydro-β-erythroidine (CLXIV), the label was shown to be specifically incorporated at positions 8 and 10 into the erythroidines as expected.

R,S-[2-^{14}C] - phenylalanine (CLX, R=phenyl) was not incorporated into the erythroidines which suggested that Erythrina species in common with most plants, cannot convert phenylalanine into tyrosine. The interpretation of this result had to be modified after it was shown by Barton[3,5] that S-N-norprotosinomenine (XIV) and not the bisphenethylamine (XXXIX) was the phenolic precursor of the aromatic Erythrina alkaloids.

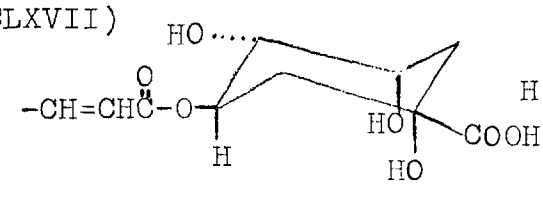


CLXIV

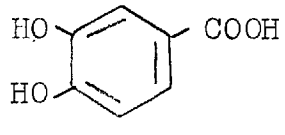


CLXV

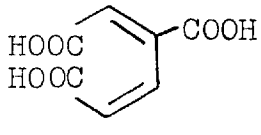
Table 4

CLXV	R_1	R_2	R_3
caffeic acid (CLXVI)	-CH=CHCOOH	H	H
chlorogenic acid (CLXVII)		H	H
ferulic acid (CLXVIII)	-CH=CHCOOH	H	CH_3
isoferulic acid (CLXIX)	-CH=CHCOOH	CH_3	H
3-(3,4-dihydroxyphenyl)-alanine (CLXX)	CH_2-CH(NH_2)COOH	H	H
2-(3,4-dihydroxyphenyl)-ethylamine (CLXXI)	$CH_2CH_2NH_2$	H	H

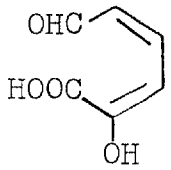
In order to try to understand oxidative ring cleavage in higher plants at the enzyme level, Finkle[65] used caffeic acid (CLXVI), chlorogenic acid (CLXVII), 3-(3,4-dihydroxyphenyl) alanine (CLXX), 2-(3,4-dihydroxyphenyl) ethylamine (CLXXI), 4'-hydroxy-3'-methoxycinnamic acid (ferulic acid, CLXVIII) and 3'-hydroxy-4'-methoxycinnamic acid (isoferulic, CLXIX) (Table 4) as model substrates for the enzymes protocatechuic acid 3,4-dioxygenase and metapyrocatechase. Both enzymes are dioxygenases[48] and catalyse the ring cleavage of 1,2-dihydroxy aromatic compounds by one molecule or two atoms of atmospheric oxygen. Protocatechuic acid 3,4-dioxygenase catalyses the intradiol cleavage of protocatechuic acid (CLXXII) to produce 3-carboxy-cis,cis- muconic acid (CLXXIII)[66,67] but it cleaves other substrates more slowly[68]. Metapyrocatechase[69,70] is an extradiol cleaving enzyme and ruptures catechol (CXLVII, R=H) to 2-hydroxy-6-oxo-hexa-2,4-cis,cis-dienoic acid (cis,cis-2-hydroxymuconic semialdehyde, CLXXIV).



CLXXII



CLXXIII



CLXXIV

In Finkles experiments the products of enzymatic catalysis were not identified but the reactions were monitored by U.V. and by uptake of molecular oxygen. It was shown that both enzymes reacted with caffeic acid (CLXVI), chlorogenic acid (CLXVII) and 2-(3,4-dihydroxyphenyl) ethylamine (CLXXI). In the case of caffeic acid one mole

of oxygen was consumed [illegible] acid 3,4-

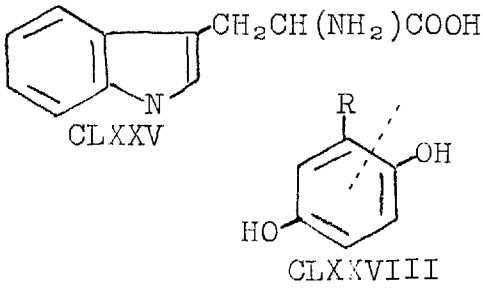
of oxygen was consumed when protocatechuic acid 3,4-dioxygenase was present. With both enzymes, ferulic acid (CLXVIII) and isoferulic acid (CLXIX) were unaffected and suggests that the 1,2-dihydroxy system is necessary for action by these enzymes.

Substrates such as CLXV when they react with meta-pyrocatechase can be cleaved in two possible extradiol positions, proximal (A) and distal (B) and Finkle's work does not differentiate between them. All the above work suggests that dioxygenases could be the enzymes responsible for aromatic ring fission in higher plants.

3. Aromatic ring fission in mammals and bacteria

This review has so far been concerned with postulated aromatic ring fission in higher plants but much more data are available on the degradation of aromatic compounds by micro-organisms. Mammalian systems have not been as well studied as micro-organisms.

During the biodegradation of tryptophan (CLXXV) in the liver, 3-hydroxyanthranilic acid (CLXXVI) has been shown[48,71] to be cleaved in the presence of a dioxygenase to 2-amino-3-carboxy-6-oxo-hexa-2,4-dienoic acid (CLXXVII).
Gentisic acid[48,72] (CLXXIX) and homogentisic acid[73] (CLXXX) were cleaved to derivatives of 4,6-dioxo-hexa-2-enoic acid (CLXXXII and CLXXXIII) in the liver.

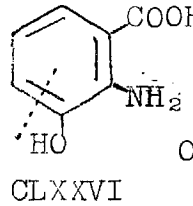


CLXXV

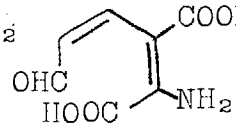
CLXXVIII

CLXXIX R=COOH

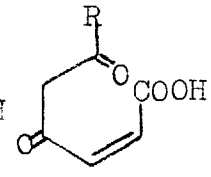
CLXXX R=CH2COOH



CLXXVI



CLXXVII



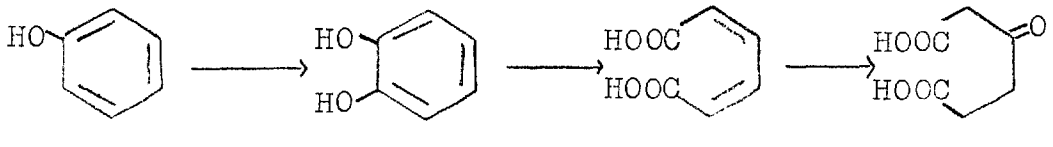
CLXXXI

CLXXXII R=COOH

CLXXXIII R=CH2COOH

For a long time it was known that micro-organisms were capable of growth on aromatic compounds as their only source of carbon.[74] Such micro-organisms which are present in soil and sewage disposal sites belong to six families, Coccaceae, Mycobacteriaceae, Bacteriaceae, Pseudomonadaceae, Spirilaceae and Bacillaceae from which the Pseudomonas species have been extensively studied. Before 1950[74], as a result of isolation experiments, it appeared that phenol (CLXXXIV) was metabolised via catechol (CLXXXV) and muconic acid (CLXXXVI) to 3-ketoadipic acid (CLXXXVII) (Scheme 11) in Pseudomonad species. Catechol (CLXXXV) could also sustain growth but when isolated muconic acid was refed to a cell free extract of Pseudomonas species by Hayaishi[75], 3-ketoadipic acid (CLXXXVII) was not produced. When Linstead[76] characterised the three geometric isomers of muconic acid (cis,cis; cis,trans; trans,trans), Hayaishi realised that isomerisation of muconic acid had occurred during work up. In 1955 the cleavage of catechol (CLXXXV) to cis,cis- muconic acid (CLXXXVI) by the enzyme, catechol 1,2-dioxygenase (pyrocatechase)[77] which was isolated from Pseudomonas species was reported.

Scheme 11

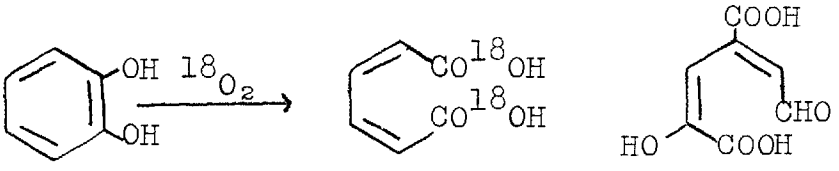


CLXXXIV CLXXXV CLXXXVI CLXXXVII

In a classic experiment, Hayaishi proved that atmospheric oxygen was the necessary oxidant.[78] Pyrocatechase was

allowed to act upon catechol in the presence of ^{18}O enriched atmospheric oxygen and a 92% incorporation of ^{18}O into the carbonyl groups of cis,cis-muconic acid was obtained. There was no incorporation of label from $H_2{}^{18}O$ in a separate experiment. (Scheme 12).

Scheme 12



CLXXXV CLXXXVIII CLXXXIX

Protocatechuic acid (CLXXII) was an intermediate postulated in the biodegradation of other aromatic compounds and it supported growth of Pseudomonas species.[74] Stanier[66] isolated protocatechuic acid 3,4-dioxygenase from P. fluorescens and it catalysed the conversion of protocatechuic acid to 3-carboxymuconic acid (CLXXIII). This dioxygenase is present in a large number of bacteria and yeast species. (Table 5).

When the intradiol method of cleavage had been established, a new mode of fission was suggested[79,80,81] since protocatechuic acid (CLXXII) in a Pseudomonas species and catechol (CLXXXV) in a 'gram negative organism' did not yield muconic acid or a derivative. The products of ring fission were later shown to be 4-carboxy-2-hydroxymuconic semialdehyde (CLXXXIX)[82,125] and 2-hydroxy-muconic semialdehyde (CLXXIV)[69] respectively. Catechol 2,3-dioxygenase (metapyrocatechase) was isolated[69] and became the

first dioxygenase to be crystallised[70]. Protocatechuic acid 4,5-dioxygenase has been isolated[83] and purified but not crystallised. Molecular oxygen is also the oxidant with the extradiol cleavage enzymes[46].

Table 5 shows the known cases of enzymatic ring cleavage. The products of these reactions can be deduced by applying the general case defined by Scheme 13.

Catechol (CLXXXV) and protocatechuic acid (CLXXII) are the most common substrates for cleavage since they are the products of degradation of such larger molecules as lipids,[124] alkylbenzene sulphonates[88,96] (CCXVI) and polyaromatics[109,110].

The chlorodihydroxybenzenes (CXCIV, CXCV, CXCVI) are intermediates in the biodegradation of 2-(4-chlorophenoxy) acetic acids (CCXVII) which are weedkillers.

7,8-Dihydroxykynurenic acid (CXCVIII) is an intermediate in the degradation of tryphotophan (CLXXV) by micro-organisms. (Scheme 14)[105] and 8-[2-(2,3-dihydroxy-6-methylphenyl)ethyl]-3,7-dioxo-4-methylhexahydroindane (CCIV) is derived from the steroid, androsta-4-ene-3,17-dione (CCXXIII) (Scheme 15).[111,112,113].

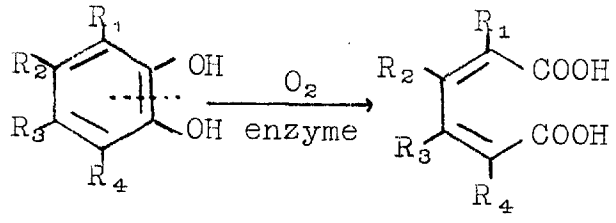
4. Pathways after ring fission

It is pertinent to consider further metabolism of the cleavage products since in all the above examples, the Stizolobium acids and betalains excepted, there is loss of at least one carbon atom. Complete degradation to acetic, pyruvic or succinic (from 3-ketoadipic acid) acids is very common.

The pathway for the formation of 3-ketoadipate (CLXXXVII)

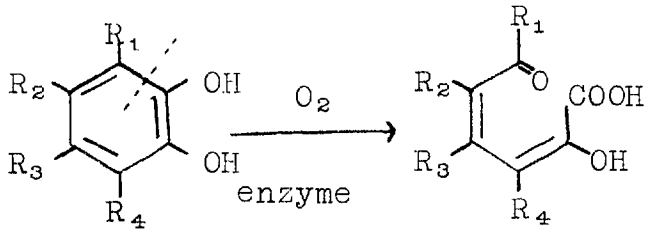
Scheme 13

Intradiol cleavage



CXC CXCI

Extradiol cleavage



CXC CXCII

Table 5

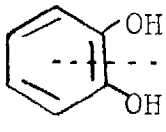
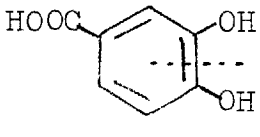
Substrate	Micro-organism	References
 CLXXXV	Pseudomonas species,[75,78] P. fluorescens[84], P. arvilla[85], Brevibacterium fuscum[86], Nocardia opaca[87], P. aeruginosa (strain B)[88], P. desmolyticum[89], Moraxella Iwoffi Vibrio (O/1)[90], Yeast species[91]	75,78, 84,85, 86,87, 88,89, 90, 91.
 CLXXII	P. fluorescens[66], Neurospora crassa[92], Nocardia erythropolis[87], Vibrio (O/1)[93,94], Azotobacter chroococum, A. vinelandii, A. beijerinckii[95], Bacillus species[96], P. desmolyticum,[89] Yeast species[91,97,98].	66,87, 89,92, 93,94, 95,96, 97,98.

Table 5 (cont.)

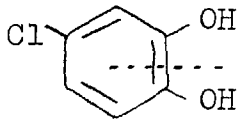
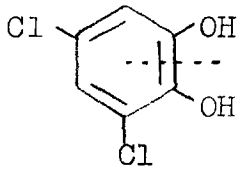
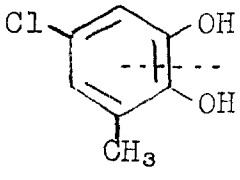
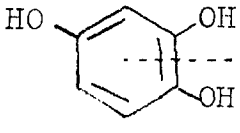
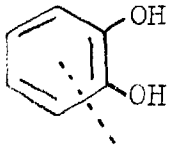
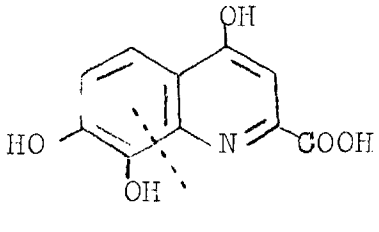
Substrate	Micro-organism	References
 CXCIV	Pseudomonadaceae[99]	99
 CXCV	Pseudomonas species[100]	100
 CXCVI	Pseudomonas species[101]	101
 CXCVII	Pseudomonas species[102]	102
 CLXXXV	'Gram negative organism'[81]; P. species[80,103], Azotobacter species[95,104].	80,81, 95,103, 104.
 CXCVIII	P. species,[105] Aerococcus[106]	105, 106.

Table 5 (cont.)

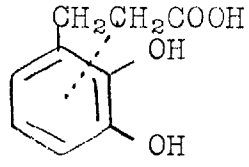
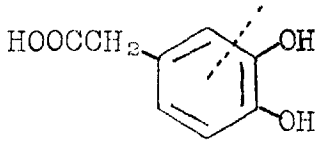
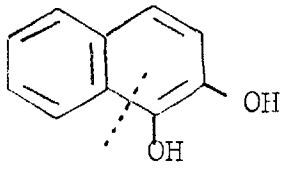
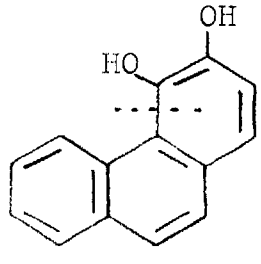
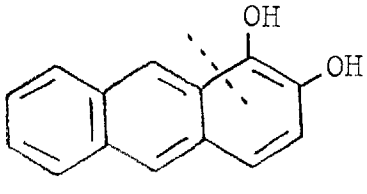
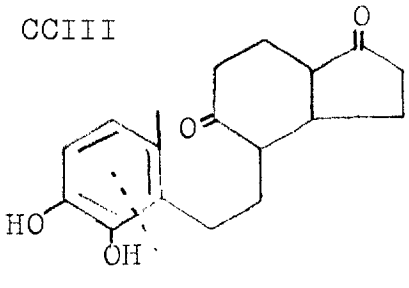
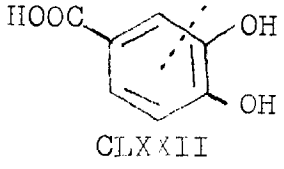
Substrate	Micro-organism	Reference
 CXCIX	Achromobacter sp.[107,108]	107,108
 CC	P. ovalis[56,57]	56,57
 CCI	P. species[109], Moraxella Iwoffi Vibrio(0/1),[90] P. aeruginosa[110]	90, 109,110
 CCII	P. aeruginosa[110]	110
 CCIII	P. aeruginosa[110]	110
 CCIV	Nocardia restrictus[111,112], N. sp.(E110)[113], P. sp., Mycobacterium smegmatis[49].	49,111, 112,113
 CLXXII	P. species[50]	50

Table 5(cont.)

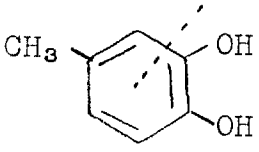
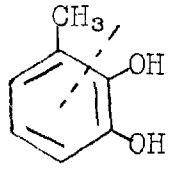
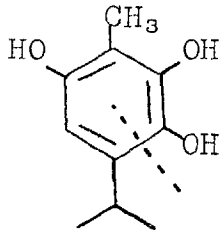
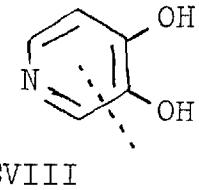
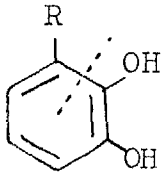
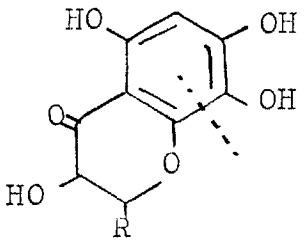
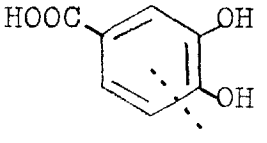
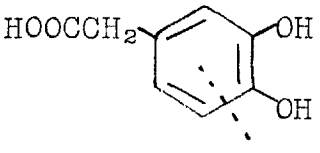
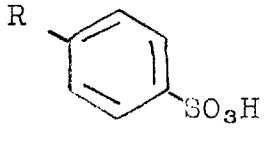
Substrate	Micro-organism	Reference
 CCV	P. species[114], P. aeruginosa[88], P. desmolyticum[89], Azotobacter sp.[104].	88,89, 104, 114
 CCVI	P. sp.[114], P. desmolyticum[89], Azotobacter sp.[104].	89,104, 114.
 CCVII	P. putida[115]	115
 CCVIII	Agrobacterium species[116]	116
 CCIX R= 3-butyl(CCX), 3-pentyl(CCXI), 4-heptyl(CCXII)	P. putida, acidovorans[117]	117

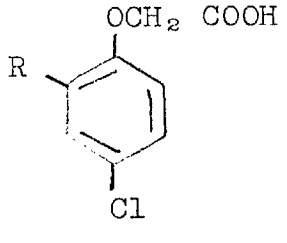
Table 5 (cont.)

Substrate	Micro-organism	References
 CCXIII R= 3,4-dihydroxyphenyl (CCXIV) R= 3,4-dimethoxyphenyl (CCXV)	P. species[118]	118
 CLXXII	P. sp.[79,80,119], Rhodopseudomonas palustris[120,122], P. testosteroni[121].	79,80, 119,120, 121,122.
 CC	P. ovalis[123]	123



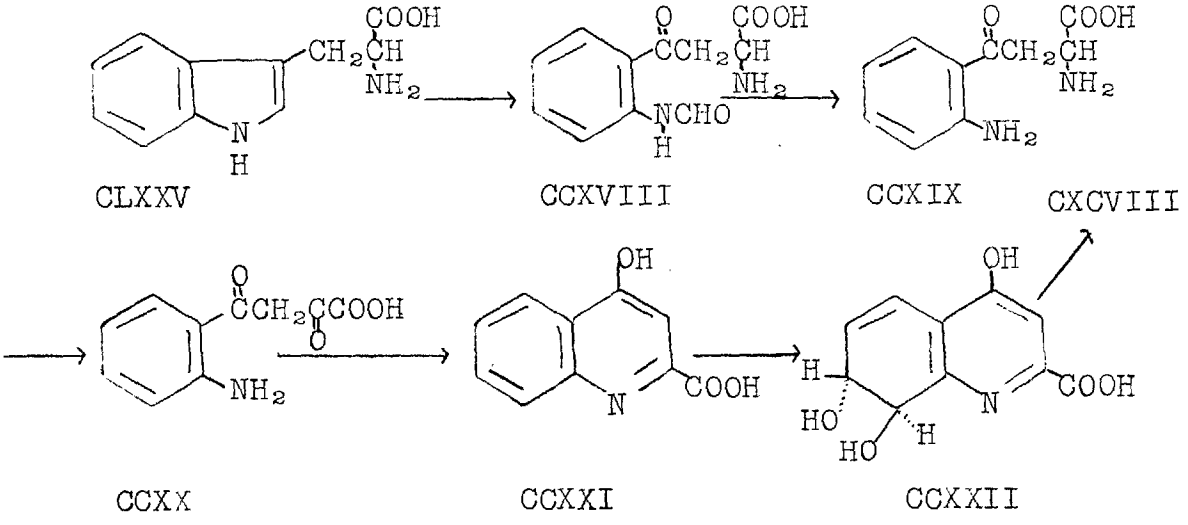
R=alkyl

CCXVI

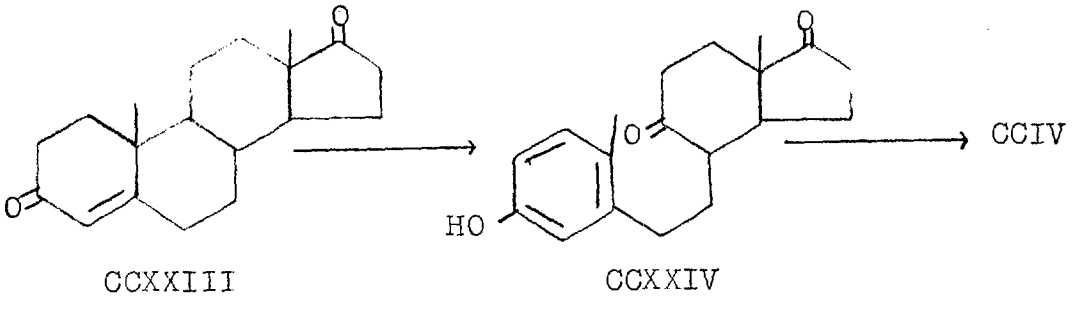


CCXVII (R=H, CH_3 or Cl)

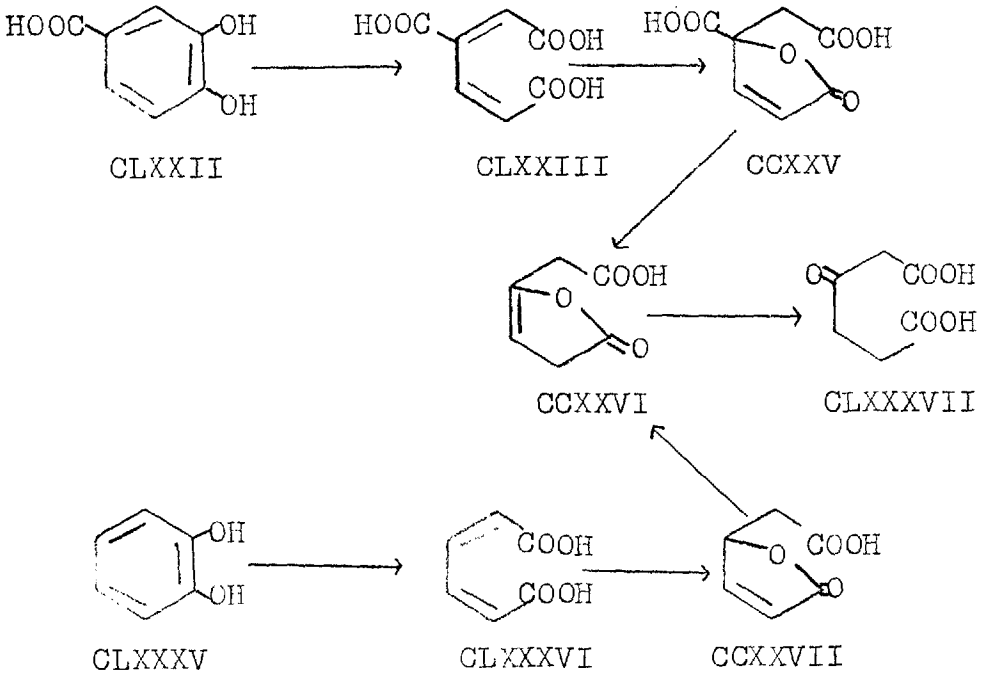
Scheme 14



Scheme 15



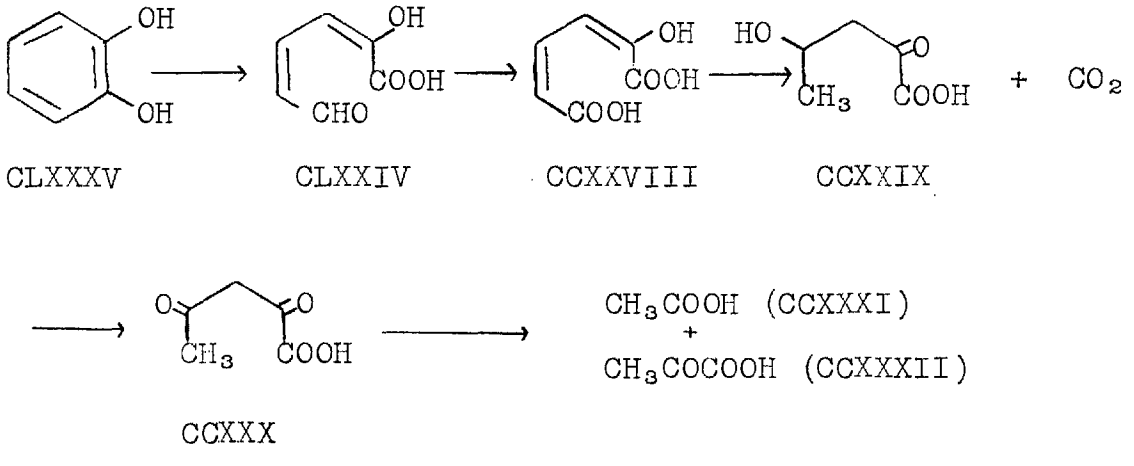
Scheme 16



from catechol (CLXXXV) and protocatechuic acid (CLXXII) after intradiol ring fission in Pseudomonads and Moraxella Iwoffi was elegantly deduced by Stanier and Ornston[94] (Scheme 16). This pathway could extend to all bacteria.

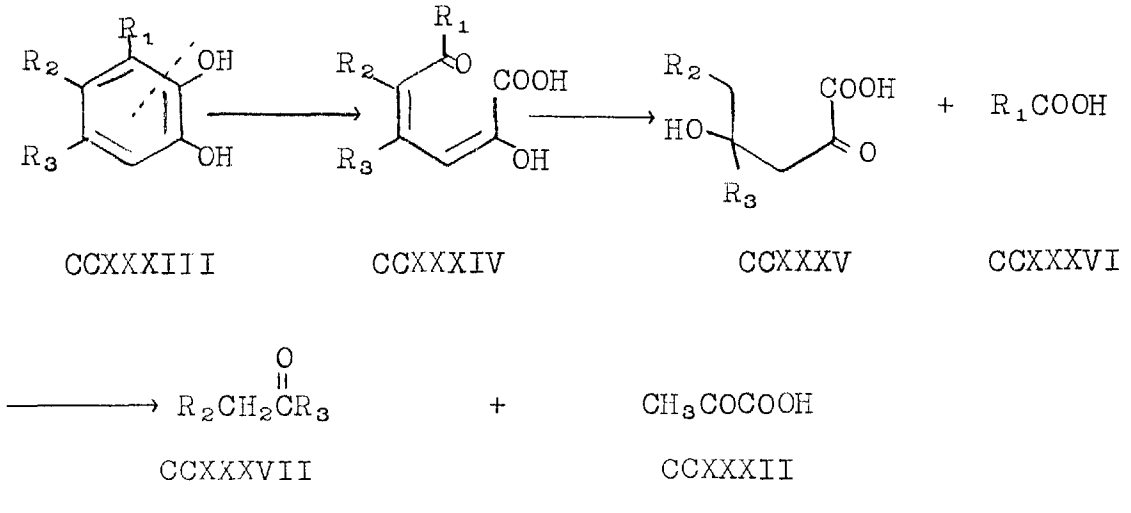
After extradiol rupture of catechol had occurred[81], Hayaishi[125] postulated that carbon dioxide, acetic acid and pyruvic acid were the final products of 2-hydroxy-muconic semialdehyde (CLXXIV) degradation (Scheme 17).

Scheme 17



Dagley and Gibson[103,114,126] proposed that the second carbon-carbon bond rupture gave formic acid from 2-hydroxymuconic semialdehyde (Scheme 18, $R_1=R_2=R_3=H$).

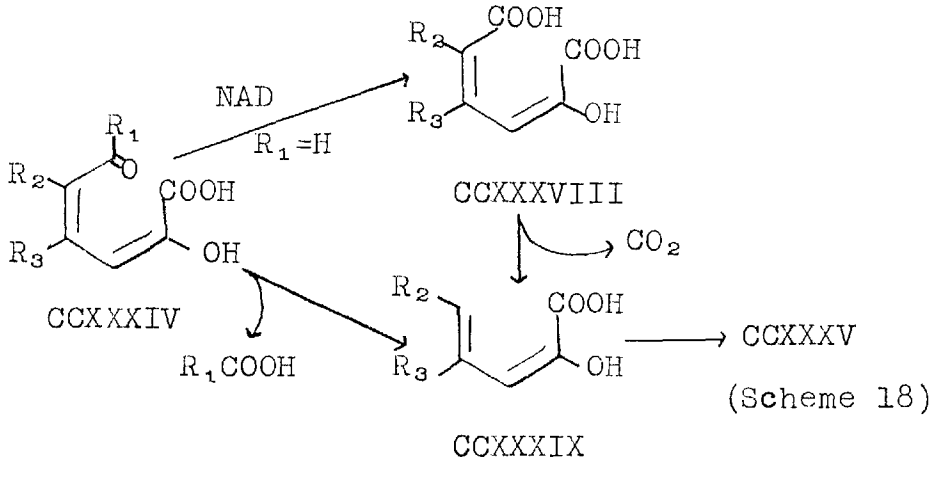
Scheme 18



From 3-methylcatechol in Pseudomonads, acetic acid, 4-hydroxy-2-oxopentanoic acid and acetaldehyde were identified (Scheme 18, R_1=CH_3, R_2=R_3=H). When 4-methylcatechol was used, formic acid, 4-hydroxy-2-oxohexanoic acid and propionaldehyde were isolated (Scheme 18, R_1=R_3=H, R_2=CH_3). This pathway was also extended to protocatechuic acid (CLXXII)[114], 3-(2,3-hydroxyphenyl) propionic acid (CXCIX)[108] (both proximal cleavage) and 2-(3,4-hydroxyphenyl) acetic acid (CC)[123] (distal cleavage).

Recent work[127] has suggested that there is a dual pathway operating in Pseudomonads grown on naphthalene. 2-Hydroxymuconic semialdehyde (CLXXIV) can act as substrate to two enzymes, one NAD dependent and the other NAD independent. These enzymes would produce (Scheme 19, R_1=R_2=R_3=H) 2-hydroxymuconic acid (CCXXVIII, Scheme 17) and 2-hydroxypenta-2,4 dienoic acid plus formic acid (c.f. Scheme 18) respectively. In Azotobacter species[104,128] the same dual pathway seemed to be operating (Scheme 19) but the NAD independent route was of only minor significance. Thus catechol gave carbon dioxide, acetaldehyde and pyruvic acid (Scheme 19, R_1=R_2=R_3=H).

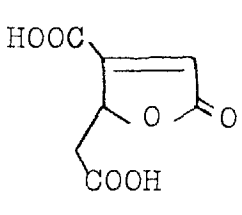
Scheme 19



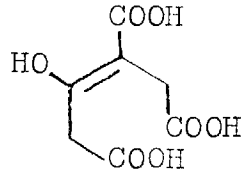
(Scheme 18)

5. Fungal aromatic cleavage and metabolism

Fungi which act upon lignin derived aromatic compounds have not been much studied. Henderson[97,98] suggested that catechol, protocatechuic acid and cis,cis-muconic acid (CLXXXVI) gave 3-ketoadipic acid (CLXXXVII) in the 'black' yeast Aureobasidium (Pullularia) pullulans and Gross[92] has demonstrated intradiol cleavage of protocatechuic acid in the fungus Neurospora crassa. Cain[91] showed that intradiol fission of protocatechuic acid and catechol to give cis,cis-3-carboxymuconic acid (CLXXIII) and cis,cis-muconic acid (CLXXXVI) respectively is identical to that observed in bacteria. (Scheme 16). The pathway, catechol to 3-ketoadipic acid, is identical in fungi and bacteria[91]. Cis,cis-3-Carboxymuconic acid from the cleavage of protocatechuic acid in fungi has been rigorously shown to give 4-carboxy-5-carboxymethyl-2-oxo-2,5-dihydrofuran (CCXL) not 5-carboxy-5-carboxymethyl-2-oxo-2,5-dihydrofuran (CCXV, Scheme 16) as in bacteria. The precise pathway of lactone (CCXL) metabolism to 3-ketoakipic acid in fungi is not known but 3-carboxy-4-hydroxy-hexa-3-ene-1,6-dioic acid (CCXLI) may be an intermediate[91].



CCXL



CCXLI

6. Enzyme Induction

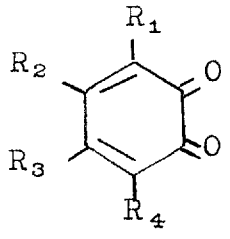
An inspection of Table 5 indicates that only monocyclic aromatic compounds undergo intradiol cleavage[49]. This phenomena may be related to the method by which the enzymes of the pathway are induced. Stanier[94] and later Hiegeman[129] suggested that intradiol fission of catechol is induced by cis,cis-muconic acid in Pseudomonas species and that extradiol cleavage is induced by phenol, cresol or an analogue. Since pyrocatechase is induced by the product of its reaction, it would be expected to be more specific than metapyrocatechase which is induced by its substrates[129]. Purification of the enzymes[77,70] indicated that metapyrocatechase has a lower substrate specificity than pyrocatechase.

7. Enzyme Mechanism of dioxygenases which act upon 1,2-dihydroxy aromatic systems.

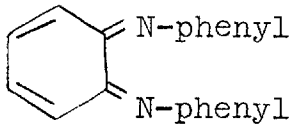
Since the discovery of dioxygenases, great advances have been made in the elucidation of their reaction mechanism; especially since some of them have been obtained in crystalline form. U.V., electron spin resonance, circular dichroism, optical rotatory dispersion and nuclear magnetic resonance techniques have all been applied with some success to this work. Complete elucidation of the mechanism, however, has so far eluded the Japanese workers led by Hayaishi.

Early speculation proposing o-benzoquinone (CCXLII) and peroxide intermediates was rejected after trapping and kinetic experiments. Aniline and catalase (an enzyme used for the assay of hydrogen peroxide) did not trap either o-

benzoquinone or hydrogen peroxide respectively[66,72]. Also, the rate of cleavage of protocatechuic acid (CLXXII) with protocatechuic acid 3,4-dioxygenase was unaffected by enzyme concentration[66].



CCXLII



CCXLIV

CCXLIII ($R_1=R_2=R_3=R_4=H$)

There is a reference[130] that o-benzoquinone anil (CCXLIV) was isolated from a trapping experiment performed on a yeast species growing on phenol. Cain[131] showed that o-benzoquinone (CCXLIII) was in fact produced non-enzymatically from catechol in yeast and that o-benzoquinone was a very efficient inhibitor of pyrocatechase and protocatechuic acid 3,4-dioxygenase. It was therefore concluded[131] that o-benzoquinone was not an intermediate.

Three dioxygenases which catalyse ring fission of 1,2-dihydroxy aromatic compounds, have been crystallised - protocatechuic acid 3,4-dioxygenase[68,83] meta pyrocatechase[70], and 3,4-dihydroxyphenylacetic acid 2,3-dioxygenase[133]. Usually greater than thirty fold purification is required before the enzyme satisfies ultracentrifugation and electrophoresis criteria for homogeneity and hence purity. Pyrocatechase[77,85], protocatechuic acid 4,5-dioxygenase[83], and 3-(2,3-dihydroxyphenyl)propionic acid 2,3-dioxygenase[134] have been highly purified but not crystallised.

Some data concerning these enzymes are given in Table 6 and a comparison of the properties of the two types of dioxygenases is included in Table 7. Hayaishi[132] showed that iron is the sole co-factor with both types of dioxygenase and that it is intimately involved in the catalytic action by interacting with the substrate. In extradiol cleaving enzymes, iron is in oxidation state two and in the pyrocatechase type enzymes iron is in the ferric state. o-Phenanthroline, a chelating agent specific for ferrous ion, inhibits meta-pyrocatechase whereas catechol-3,5-disulphonic acid, a potent chelating agent for ferric ion, has no effect.

An e.s.r. signal at g=4.28 has been attributed to ferric ion in pyrocatechase. This signal[132,138] disappeared in the presence of catechol then reappeared when oxygen was introduced and cis,cis-muconic acid exhausted. A similar effect was shown with protocatechuic acid 3,4-dioxygenase[139]. With both enzymes, the U.V. spectra showed corresponding changes and a spectral species was observed on the addition of substrate which was assigned to a substrate: iron:enzyme complex. Furthermore for protocatechuic acid 3,4-dioxygenase, an additional spectral species was observed in the presence of substrate, oxygen and enzyme[136]. After analysis of stopped flow kinetics between less active substrates (e.g. 2,3-dihydroxyphenylacetic acid) and this enzyme[136,140], it was proposed that the new spectral species was a terniary complex of oxygen, substrate and enzyme[139,140].

When catechol was oxygenated using pyrocatechase and only ^{18}O-^{18}O and ^{16}O-^{16}O species of oxygen, the two carboxyl groups of cis,cis-muconic acid had obtained their oxygen atom

Table 6

Properties of some dioxygenases

Enzyme	Molecular weight	Number of atoms of iron per molecule of enzyme
Pyrocatechase	95000[135], 78000[86]	2[135]
Protocatechuic acid 3,4-dioxygenase	700000[136]	8[136]
Metapyrocatechase	140000[70]	1[139]
Protocatechuic acid 4,5-dioxygenase	140000[121]	1[83]
3-(2,3-Dihydroxyphenyl)propionic acid 1,2-dioxygenase	140000[134]	
3,4-Dihydroxyphenylacetic acid 2,3-dioxygenase	100000[133]	4-5[133]

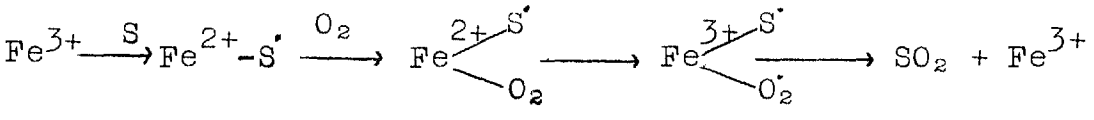
Table 7

Comparison of extradiol and intadiol cleaving dioxygenases[132]

Property	Extradiol	Intradiol
colour	colourless	red
oxidising agents	inactivate	no effect
reducing agents	no effect	inactivate
o-phenanthroline	inhibit	no effect
pre-incubation with catechol 3,5-disulphonic acid under anaerobic conditions	no effect	inactivate
e.s.r. signal at g=4.2	absent	present
e.s.r. signal at g=4.2 in the presence of substrate	appears	disappears

from the same molecule. This and other data supports the mechanism proposed by Hayaishi[138,141] for intradiol cleaving dioxygenases (Scheme 20).

Scheme 20

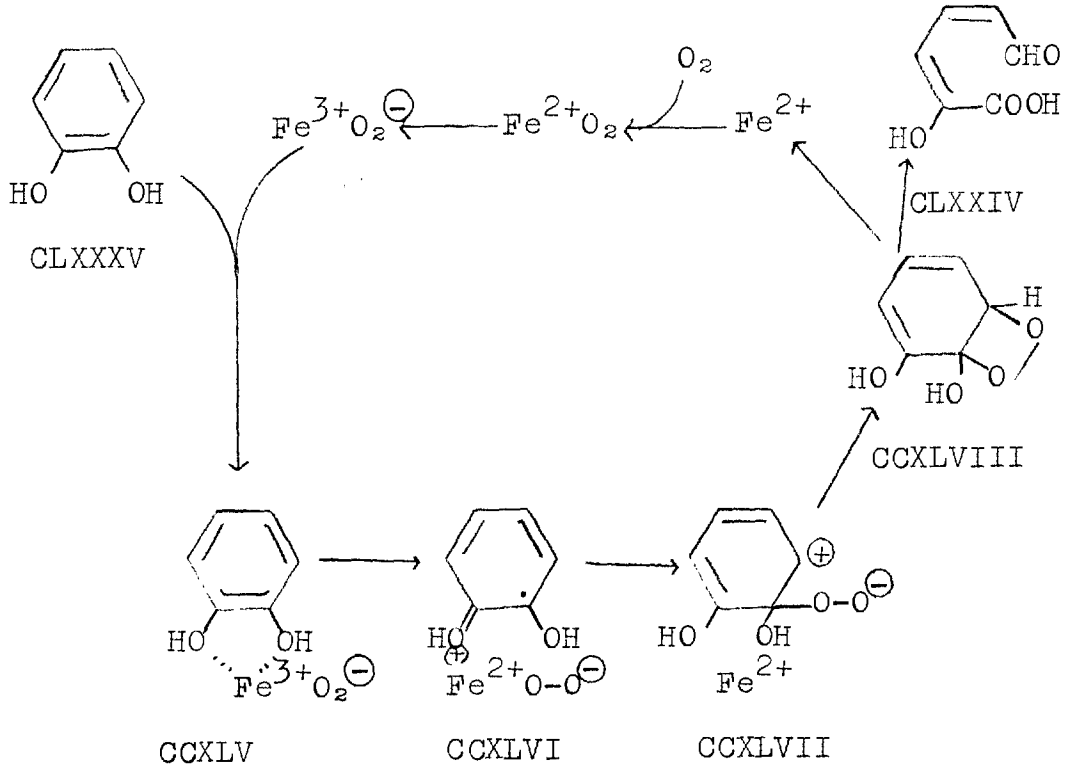


S = substrate

Although the oxidation state of iron in extradiol cleaving dioxygenases is different from the pyrocatechase type, the proposed reaction mechanism[132] is similar to that above since a terniary complex of substrate, iron and oxygen during the course of catalysis, is postulated in both cases.

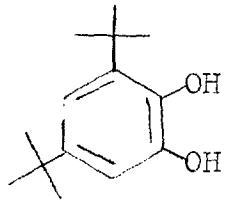
Hayaishi with metapyrocatechase[132] and Senoh with 3,4-dihydroxyphenylacetic acid 2,3-dioxygenase[133] using e.s.r. and U.V. proposed that a ferric ion:substrate:oxygen complex is formed. In this case the e.s.r. signal at g=4.2 (due to iron (III)) appeared only in the presence of substrate, enzyme and oxygen. Initial rate studies with 3-(2,3-dihydroxyphenyl) propionic acid 1,2-dioxygenase also suggested a terniary complex[134]. The mechanism of all extradiol cleaving dioxygenases may be identical to that tentively postulated by Hayaishi[142] (Scheme 21).

Scheme 21

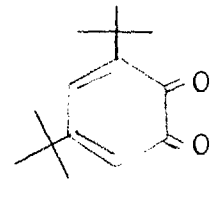


8. Cleavage of aromatic rings by analagous chemical means.

Catechol can be cleaved to cis,cis-muconic acid by peracetic acid[76] and o-benzoquinone (CCXLIII) is oxidised to the same product with hydrogen peroxide[143]. Grinstead[144] proposed that enzymatic intradiol fission proceeded via enzyme bound o-benzoquinone and hydrogen peroxide when 3,5-di-t-butylcatechol (CCXLIX) was oxidised to o-quinone (CCL) in the presence of metal ions (e.g. Fe^{2+}, Co^{2+}, Zn^{2+}) and oxygen.



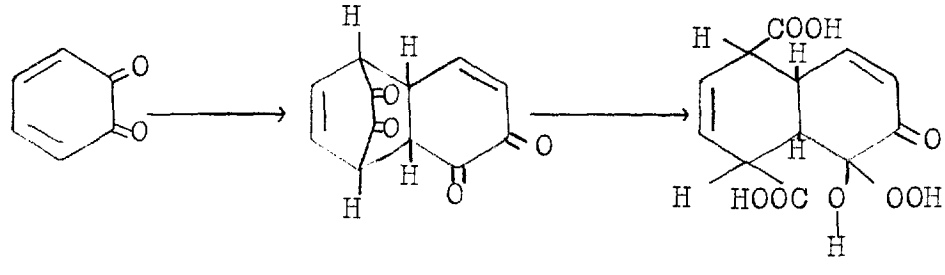
CCXLIX



CCL

Patchett[143] suggested that a peroxy intermediate could be involved when structure (CCLII, Scheme 22) was isolated from the hydrogen peroxide oxidation of o-benzoquinone (CCXLIII). The peroxy compound (CCLII) was derived from the Diels- Alder dimerisation product of o-benzoquinone (CCLI) by oxidative ring cleavage and attack by the peroxide anion.

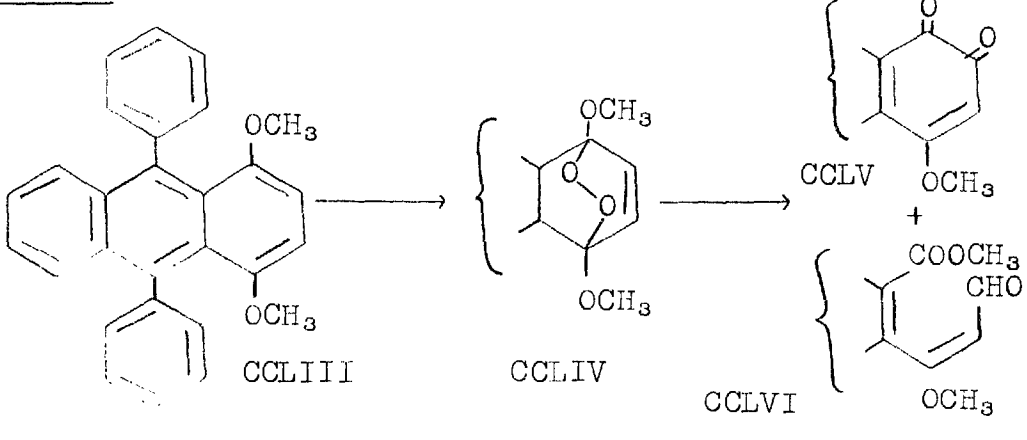
Scheme 22



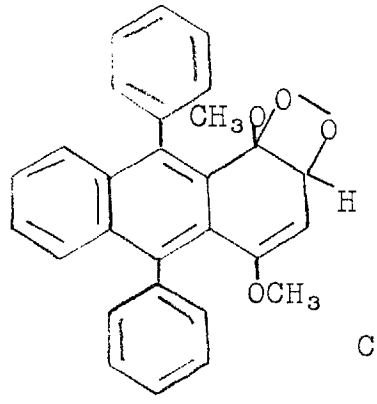
CCXLIII CCLI CCLII

Baldwin[145] produced ring fission by re-arrangement of 1,4-peroxide (CCLIV) and suggested that singlet oxygen could be involved. The existence of cyclic peroxide (CCLVII) as an intermediate, however, was not precluded. 1,4-Peroxide (CCLIV) was synthesised by photolytic oxygenation of (CCLIII) and the rearrangement was performed with ethereal hydrogen chloride. (Scheme 23).

Scheme 23



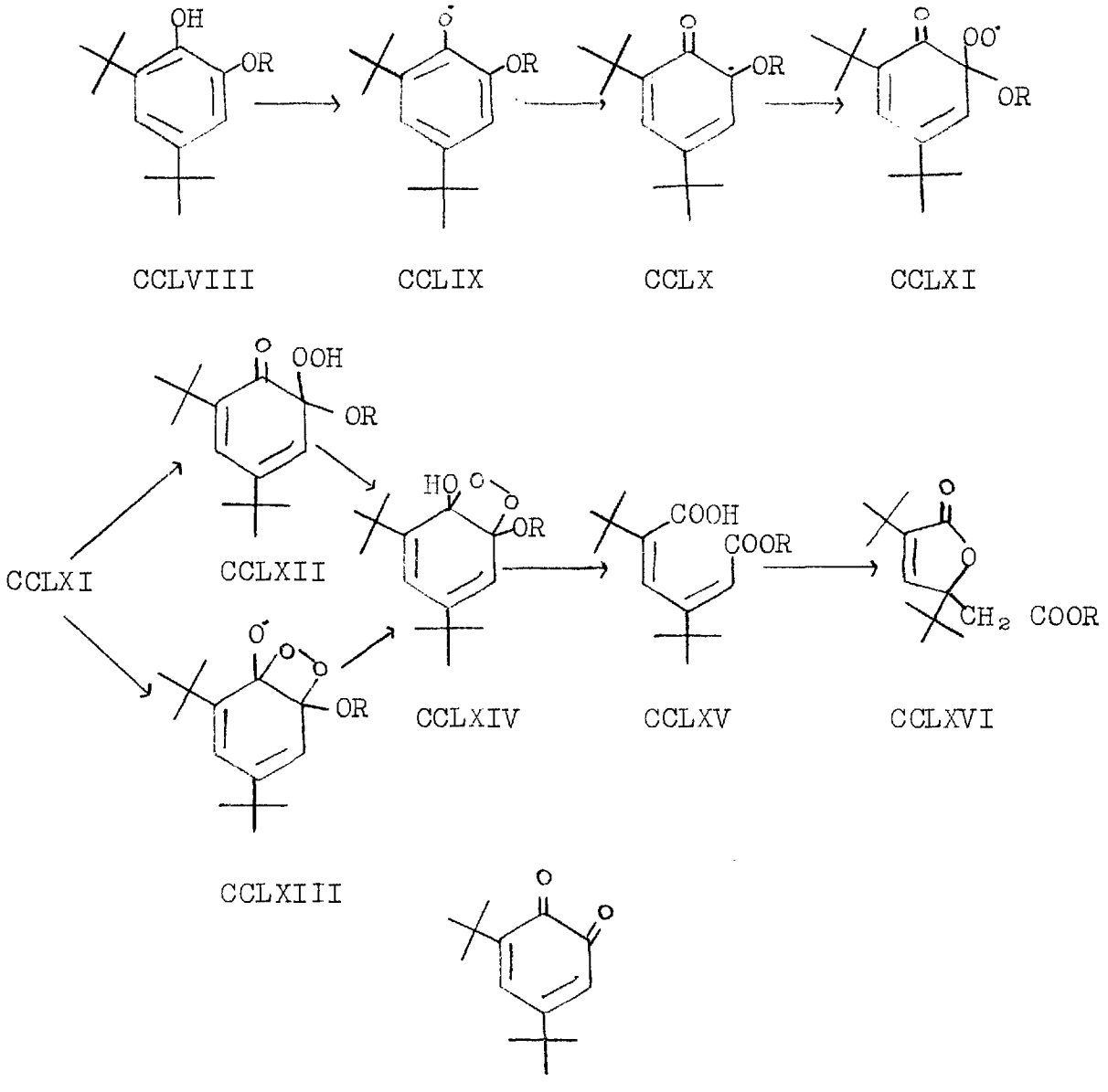
CCLIII CCLIV CCLV CCLVI



CCLVII

Some very recent work[146] on the photooxidation of 3,5-di-t-butyl catechol (CCXLIX) has provided more evidence against an o-quinone intermediate in the enzymatic catalysis. The product (Scheme 24) in pyridine was (3,5-di-t-butyl-2-oxo-1,5-dihydro-5-furanyl)acetic acid (CCLXVI) or the lactone acid (CCLXVI) and its ester (CCLXVI, R=CH_3) in methanol. The lactone (CCLXVI) is directly analagous to the lactones (CCXXV and CCXXVII, Scheme 16) proposed in the bacterial intradiol degradation in Pseudomonas species of protocatechuic acid and catechol respectively. During photooxidation (Scheme 24), a hydrogen atom is removed by the radiation, sensitisor or singlet oxygen and the rearranged radical (CCLX) combines with triplet oxygen to give the intermediate (CCLXI). The peroxyradical intermediate (CCLXI) can rearrange either before or after combining with a hydrogen atom to give the cyclic peroxide (CCLXIV) which cleaves and recyclises to give the unsaturated lactone (CCLXVI). When 3,5-di-t-butyl-o-benzoquinone (CCLXVII) was photolysed, the reaction was much slower, more complex and the product ratio different from that above.

Scheme 24



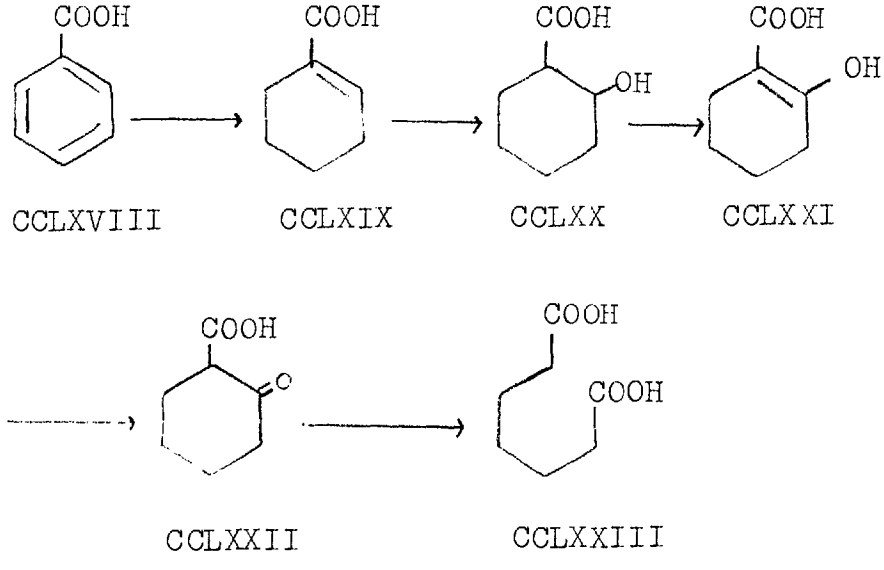
Although o-quinone (CCLXVII) may be a source of product it was concluded that this was only a minor route.

9. Reductive Cleavage Mechanism.

All the mechanisms mentioned in this review have involved oxidative cleavage of an aromatic ring. There has,

however, been one instance in the literature where initial reduction of the aromatic ring was postulated as the first step[122]. Evans proposed that benzoic acid was degraded to hepta-1,7-dioic acid when fed to Rhodopseudomonas palustris and photolysed anaerobically. (Scheme 25).

Scheme 25



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DISCUSSION

1. Comparative feeding of tetrahydroisoquinoline precursors to Erythrina species.

2. Feedings to the erythroidines in E. berteroana.

3. Alkaloid metabolism in E. berteroana.

4. Other feeding methods with Erythrina species.

5. Variation of alkaloid content with time in E. crista galli seedlings.

6. Isolation and partial structure of the unknown alkaloid.

EXPERIMENTAL

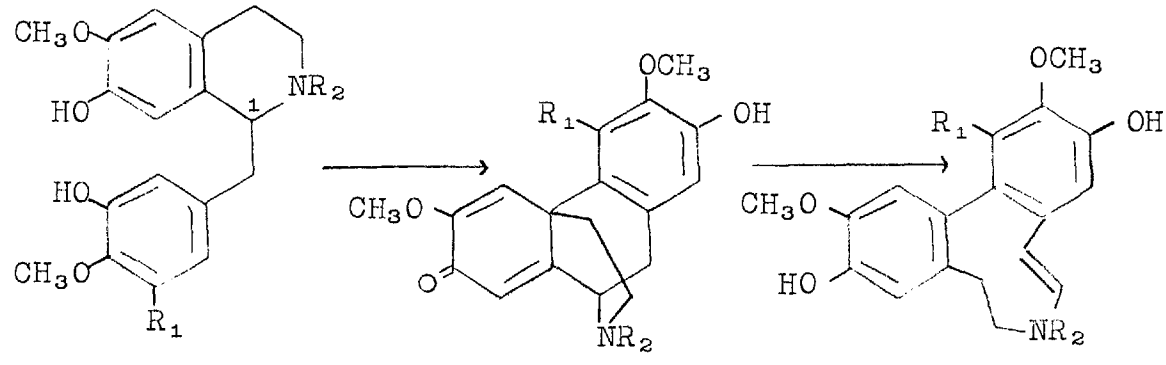
REFERENCES

DISCUSSION

1. Comparative feeding of tetrahydroisoquinoline precursors to Erythrina species.

Franck[1] suggested that N-norreticuline (III) could be a biogenetic precursor of the aromatic Erythrina alkaloid skeleton after he was able to synthesise 14-methoxyerysodienone (XIV) from N-mesyl-5'-methoxy-N-norreticuline (II) (Scheme 1. $R_1=OCH_3$, $R_2=SO_2CH_3$). Oxidation of N-mesyl-5'-methoxy-N-norreticuline using vanadium (III) oxytrichloride gave the morphadienone derivative (V) in 34% yield by p-p coupling of the phenolate radicals.[2] Treatment with boron trifluoride produced a dienone-phenol rearrangement and aromatisation to give 8,9-dihydro-1,2,12-trimethoxy-7-mesyldibenz[d,f]azonine-3,11-diol (VIII) in 63% yield. Reduction with hydrogen over platinum (81% yield) and removal of the protecting group with lithium in ammonia (72%) gave 5,6,8,9-tetrahydro-1,2,12-trimethoxy-7H-dibenz[d,f]azonine-3,11-diol (XI). The biphenyl (XI) was oxidised to 14-methoxyerysodienone (XIV) using alkaline potassium ferricyanide in 61% yield. The proposed pathway in vivo would presumably follow Scheme 1. $R_1=R_2=H$.

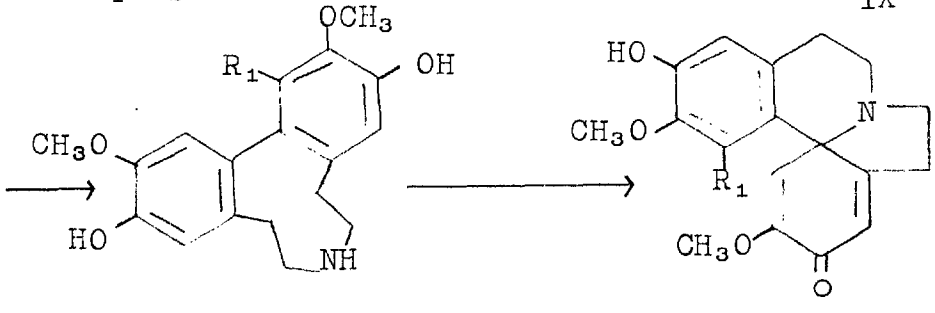
Scheme 1



I IV VII

II R_1=OCH_3, R_2=SO_2CH_3 V R_1=OCH_3, R_2=SO_2CH_3 VIII R_1=OCH_3, R_2=SO_2CH_3

III R_1=R_2=H VI R_1=R_2=H IX R_1=R_2=H



X XIII

XI R_1=OCH_3 XIV R_1=OCH_3

XII R_1=H XV R_1=H

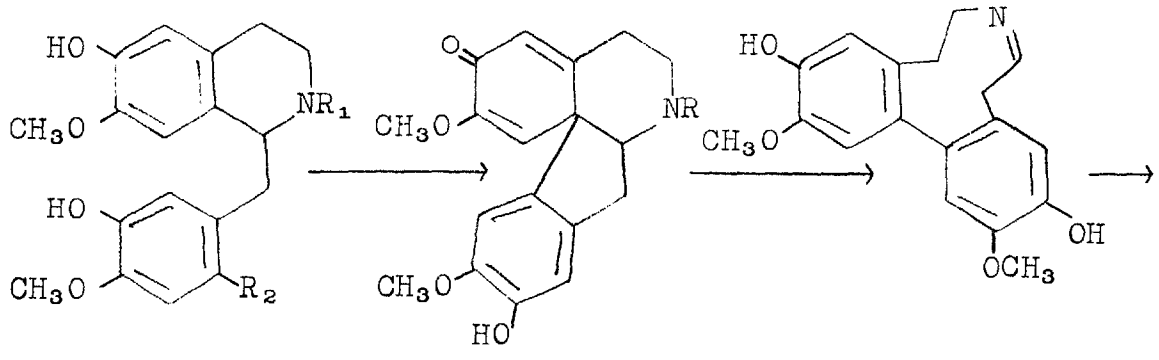
Table 1

Stereochemistry and position of the label in N-norprotosinomenine (XVII)	$^3H/^{14}C$ ratio of precursors	Incorporation into erythraline /%	$^3H/^{14}C$	^{14}C label $O\underline{C}H_2O$	^{14}C label $O\underline{C}H_3$
(±) [5,2',6'-$^3H_3$]	-	0.048[3]	-	-	-
(±) [5,2',6'-$^3H_3$]	-	0.24[3]	-	-	-
S-(XXVII) [5-^3H]	-	0.21[4]	-	-	-
	-	0.12[4]	-	-	-
R-(enantiomer XXVII)[5-^3H]	-	0.002[4]	-	-	-
(±)[3-^{14}C,5-^3H]	2.04	0.087[4]	1.98	-	-
(±)[4'-methyl-^{14}C]	-	0.25[4]	-	52.5	49.6

Barton and co-workers had earlier[3] proposed that N-norprotosinomenine (XVII) was the tetrahydrobenzylisoquinoline precursor of the aromatic Erythrina alkaloids and (±)-[5,2',6'-$^3H_3$]-N-norprotosinomenine was incorporated into erythraline (XXVI) in E. crista galli plants. The resolution of N-norprotosinomenine was accomplished and parallel feedings of R and S enantiomers showed that only the S isomer (XXVII) was incorporated[4]. This would be expected for the operation of an enzymatic system. The results of all feedings of single and double labelled N-norprotosinomenine are given in Table 1. and the pathway to the key intermediate, erysodienone (XV) is set out in Scheme 2 (R_1=R_2=H).

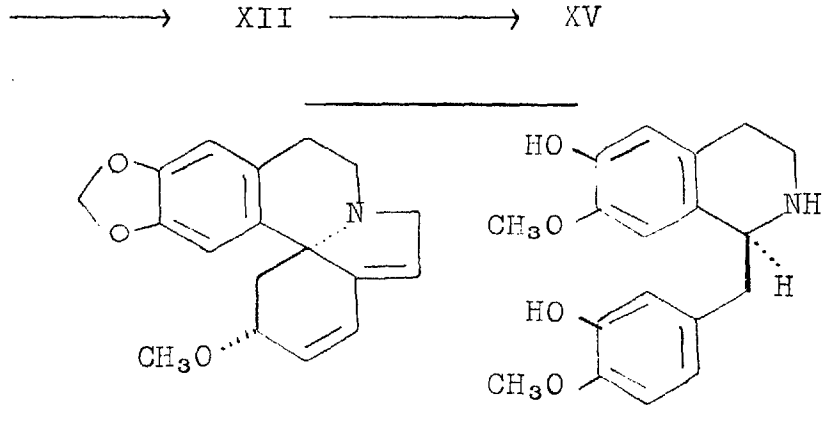
Kametani[5] has reproduced in vitro the first oxidative step of Scheme 2. N-Ethoxycarbonyl-N-norprotosinomenine (XVIII) was oxidised in the presence of potassium ferricyanide in a pH 9 buffer of ammonia/ammonium acetate in 2% yield to the proerysodienone (XXIII) which in the presence of concentrated sulphuric acid rearranges to 5,6,7a,8-tetrahydro-3,10-dihydroxy-11-methoxy-2-oxo-dibenz[d,f]indole-7 (2H)-carboxylic acid ethyl ester (XXIX) (Scheme 3). The yield has recently been improved by using a photolytic method.[6] 2'-Bromo-N-ethoxycarbonyl-N-norprotosinomenine (XIX) gives the proerysodienone (XXIII) in 12% yield whilst 2'-bromo-N-trifluoroacetyl-N-norprotosinomenine (XX) gives XXIV in 4% yield. N-Trifluoroacetyl-proerysodienone (XXIV) was characterised as its rearranged product (XXX) (Scheme 3) with concentrated sulphuric acid.

Scheme 2



XVI XXI XXV

	R_1	R_2		
XVII	H	H	XXII	R=H
XVIII	COOEt	H	XXIII	R=COOEt
XIX	COOEt	Br		
XX	$COCF_3$	Br	XXIV	$R=COCF_3$

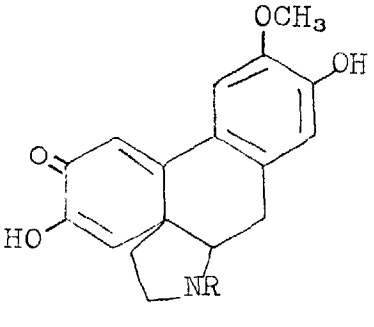


XII XV

XXVI XXVII

Scheme 3

XXIII
XXIV

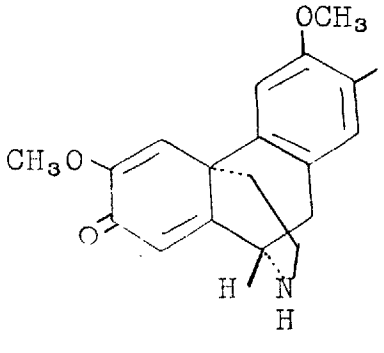


XXVIII

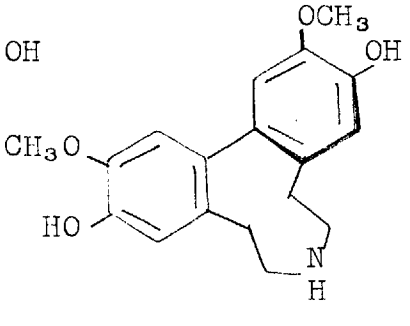
XXIX R=COOEt

XXX $R=COCF_3$

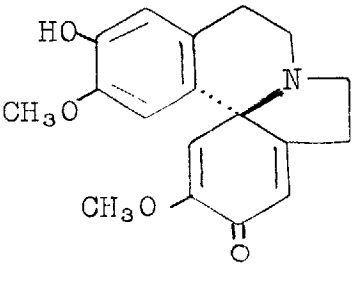
The N-norprotosinomenine feeding results outlined in Table 1 do not preclude a possible pathway of S-N-norprotosinomenine (XXVII) giving S-N-norreticuline (III, α-C_1-H) by demethylation and remethylation steps. Phenol oxidative coupling (Scheme 1) of S-N-norreticuline (II, α-C_1-H) would give the morphadienone (XXXI). A dienone-phenol rearrangement and aromatisation on the chiral surface of an enzyme would produce 8,9-dihydro-2,12-dimethoxy-7H-dibenz [d,f]azonine-3,11-diol which without racemisation would reduce to 5,6,8,9-tetrahydro-2,12-dimethoxy-7H-dibenz [d,f] azonine-3,11-diol (XXXII). The stereochemistry of the dibenz azonine (XXXII) would be the same as that derived from S-N-norprotosinomenine[4]. Oxidation would produce 5R-erysodienone (XXXIII) which has the opposite configuration to that of the natural alkaloids (c.f. erythraline XXVI). Consequently, if S-N-norreticuline is a genuine precursor of 5S-erysodienone, some inversion or symmetrising process must occur.



XXXI



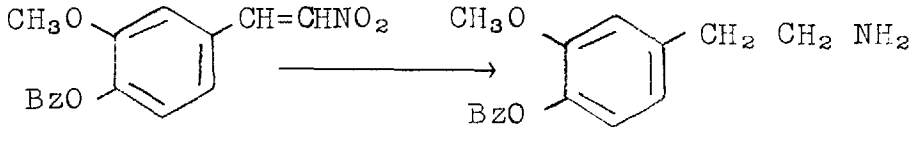
XXXII



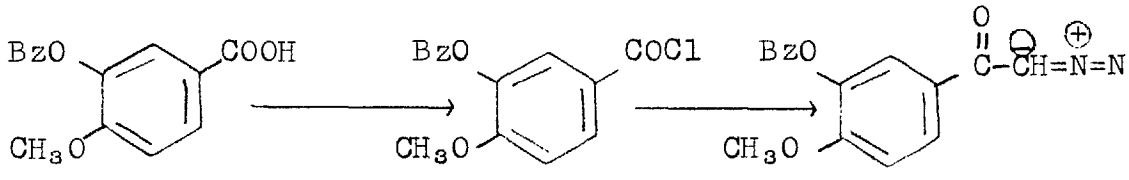
XXXIII

N-Norreticuline was synthesised by well-known procedures (Scheme 4). 1-Nitro-2-(4-benzyloxy-3-methoxyphenyl)-ethylene (XXXIV) was reduced by lithium aluminium hydride in refluxing tetrahydrofuran to 2-(4-benzyloxy-3-methoxyphenyl) ethylamine[7] (XXXV) in 70% yield. 3-Benzyloxy-4-methoxy benzoic acid (XXXVI)[8] was converted to the acid chloride using thionyl chloride in benzene and after removal of the benzene, the acid chloride with diazomethane in ether at 5° gave 3-benzyloxy-4-methoxy-w-diazoacetophenone (XXXVIII) in 90% yield. 2-(4-Benzyloxy-3-methoxyphenyl) ethylamine (XXXV) and 3-benzyloxy-4-methoxy-w-diazoacetophenone (XXXVIII) were photolysed[8] in dry benzene for 3 hour with a 125w high pressure mercury lamp, and N-[-2-(4-benzyloxy-3-methoxyphenyl)ethyl]-2-(3-benzyloxy-4-methoxyphenyl) acetamide (XXXIX) was obtained in 61% yield. Using a Bischler-Napieralski synthesis[9], the amide (XXXIX) was cyclised in dry refluxing toluene with phosphorusoxychloride to 7-benzyloxy-1-(3-benzyloxy-4-methoxybenzyl)-3,4-dihydro-6-methoxyisoquinoline hydrochloride (XL) in 62% yield. Reduction of the imine with sodium borohydride and removal of the benzyl protecting groups with hot concentrated hydrochloric acid yielded N-norreticuline (III) as its hydrochloride salt. A portion of N-norreticuline was subjected to base catalysed exchange with tritiated water[3]. The crude product was separated on t.l.c. to give R,S-7-hydroxy-1-(3-hydroxy-4-methoxy-[2,6-$^3H_2$]-benzyl)-6-methoxy-1,2,3,4-tetrahydro-[8-^3H]-isoquinoline (XLII)([aryl-^3H]-N-norreticuline). A duplicate experiment using deuterium oxide indicated the loss of 3 aromatic protons o and p to the hydroxy groups.

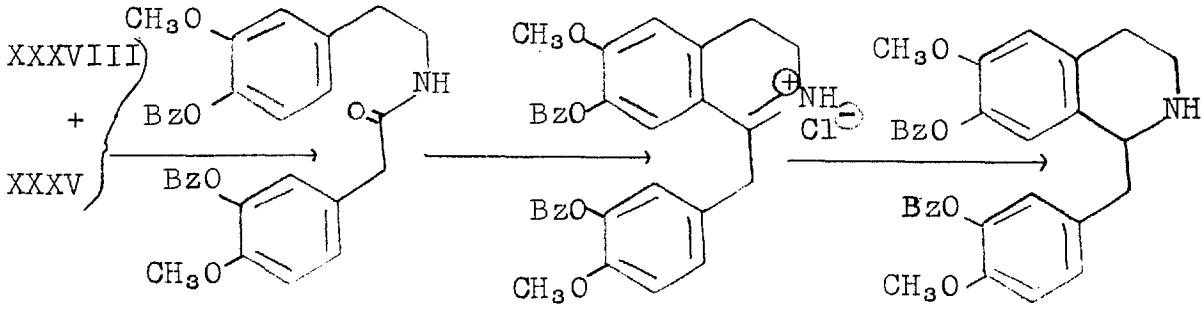
Scheme 4



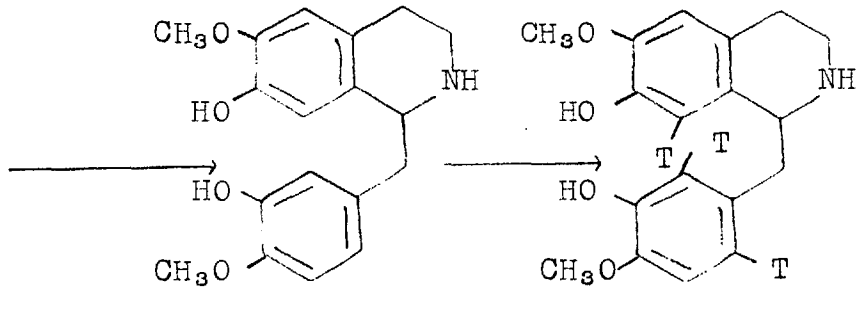
XXXIV XXXV



XXXVI XXXVII XXXVIII

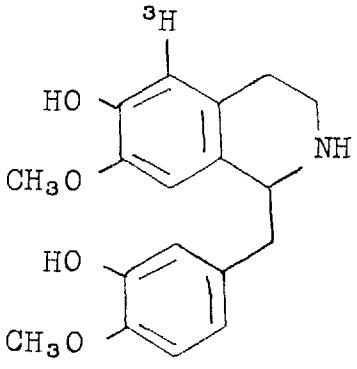


XXXIX XL XLI

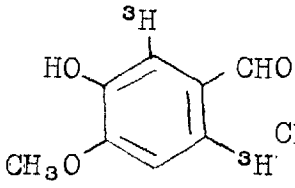


III XLII

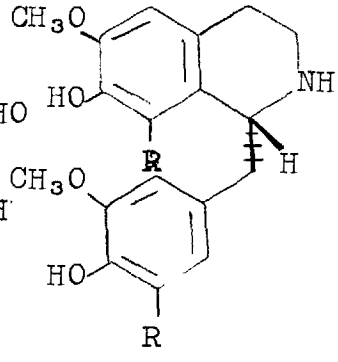
R,S-[5-^{3}H]-N-norprotosinomenine (XLIII) had been prepared by previous workers from [2,6-$^3H_2$]-isovanillin (XLIV)[4]. This compound was purified before feeding.



XLIII



XLIV



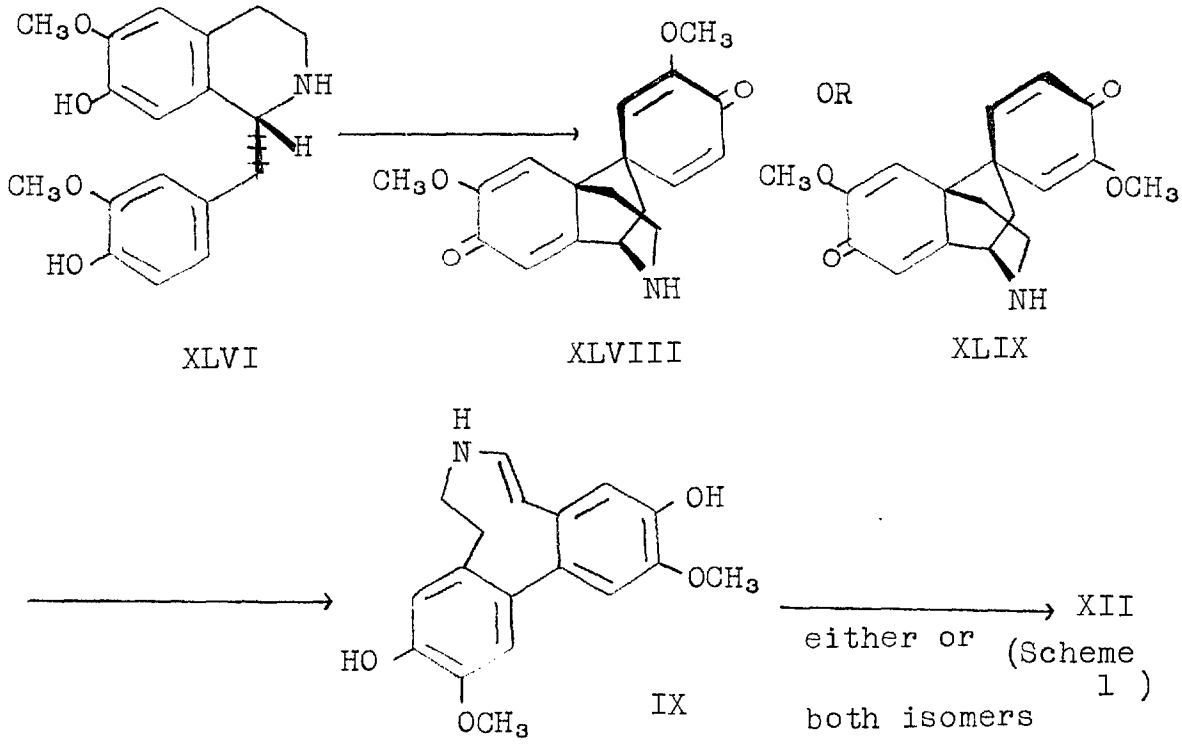
XLV

XLVI R=H

XLVII R=^{3}H

A colleague, Miss A. Lobo, had isolated R-(+)-N-nororientaline (XLVI) from Erythrina poeppigiana[10] and this was subjected to base catalysed exchange with tritiated water. A similar exchange with deuterium oxide indicated the loss of two aromatic protons. Since positions 8 and 5' are o to hydroxy groups, R-[aryl-^{3}H]-N-nororientaline is therefore R-7-hydroxy-1-(4-hydroxy-3-methoxy-[5-^{3}H]-benzyl)-6-methoxy-1,2,3,4-tetrahydro-[8-^{3}H]-isoquinoline (XLVII). A pathway which gives the tetrahydrobenz[d,f]azonine (XII, Scheme 1) from R-N-nororientaline would involve two dienone-phenol rearrangements. (Scheme 5). P-p Phenol oxidative coupling of R-N-nororientaline on an enzyme surface would produce one of two possible isomers (XLVIII or XLIX).

Scheme 5



R,S-[2-^{14}C]-Tyrosine (L), R,S-[aryl-^{3}H]-N-norreticuline (XLII), R,S-[5-^{3}H]-N-norprotosinomenine (XLIII) and R-[aryl-^{3}H]-N-nororientaline (XLVII) were fed at the same time as aqueous solutions of their hydrochloride salts to Erythrina crista galli plants (Table 2). Each precursor was fed to two healthy first year plants (5 months old) using the wick feed method. The whole plant was sacrificed, macerated and extracted for crude alkaloid with 0.06M-hydrochloric acid. Inactive erythraline (XXVI) was added. Separation on t.l.c. gave erythraline which was crystallised and counted as its hydrobromide salt. Reseparation on t.l.c. gave no loss of activity.

Table 2

Parallel feedings to E. crista galli in 1972 season

The plants were fed on 26/5/72 and harvested on 6/6/72 (11 days)

Precursor	Activity fed /10^4dps	Incorporation into erythraline /%
R,S-[2-^{14}C]-tyrosine(L)	19.2	0.18±0.02
R,S-[5-^{3}H]-N-norprotosinomenine(XLIII)	5.1	0.051±0.005
R,S-[aryl-^{3}H]-N-norreticuline(XLII)	21.0	0.001
R-[aryl-^{3}H]-N-nororientaline(XLVII)	22.5	$<10^{-4}$

Table 3

Parallel feedings to E. berteroana in 1972 season

Plants were fed on 26/5/72 and harvested on 6/6/72. (11 days)

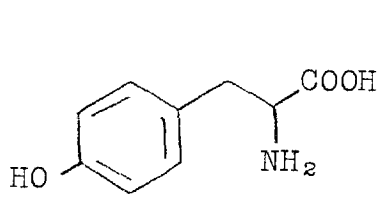
Precursor	Activity fed /10^4dps	Incorporation into erythroidine /%
R,S-[2-^{14}C]-tyrosine(L)	19.2	0.002
R,S-[5-^{3}H]-N-norprotosinomenine(XLIII)	5.40	$<10^{-4}$

The feedings of R,S-[aryl-^{3}H]-N-norreticuline and R-[aryl-^{3}H]-N-nororientaline were not worked up.

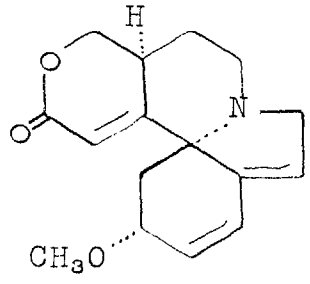
Table 4 (E. berteroana)

Plants fed on 26/5/72 and harvested on 28/6/72 (4weeks 5days)

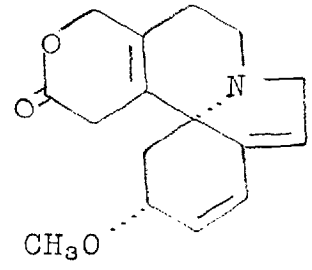
Precursor	Activity fed /10^4dps	Incorporation into erythroidine /%
R,S-[2-^{14}C]-tyrosine(L)	19.2	0.032±0.002
R,S-[5-^{3}H]-N-norprotosinomenine(XLIII)	5.0	0.060±0.005
R,S-[aryl-^{3}H]-N-norreticuline(XLII)	25.5	0.008±0.0005
R-[aryl-^{3}H]-N-nororientaline(XLVII)	25.0	$<10^{-3}$



L



LI



LII

The four precursors were also fed in parallel to E. berteroana plants (5 months old). Two groups of plants were used, one was harvested after 11 days (Table 3) and the other after 4 weeks 5 days (Table 4). Each precursor was fed to a single E. berteroana plant using the wick feed method. The whole plant was harvested, macerated and extracted for crude alkaloid which was diluted with inactive α-erythroidine (LI). Separation on t.l.c. gave a mixture of α-erythroidine (LI) and β-erythroidine (LII)[11] which was inseparable on alumina (50% ethyl acetate-benzene). They were also not clearly distinguishable on silica (5% methanol-chloroform)[12] even after multiple elution. It has been demonstrated[13] that α-and β- erythroidine hydrochlorides co-crystallise and so the mixture was recrystallised to constant activity. Reseparation of the α-/β-erythroidine mixture from the R,S-[5-^{3}H]-N-norprotosinomenine feeding on t.l.c. gave no change in the specific activity.

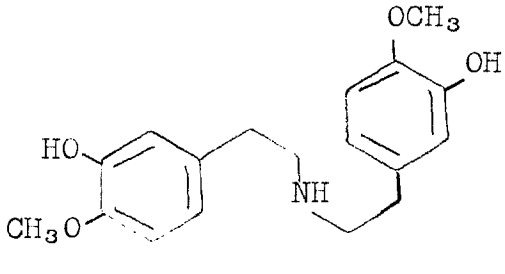
The incorporations from the feeding of R,S-[aryl-^{3}H]-N-norreticuline have been corrected for the loss of one tritium atom per molecule (from the 6' position).

The incorporations of tyrosine agree well with those

obtained by previous workers (0.121%[3] into erythraline and 0.025%[12] into the erythroidines) using the same Erythrina species. Comparison of Tables 1 and 2 reveals that the incorporation of R,S-N-norprotosinomenine into erythraline in E. crista galli is acceptable. R,S-N-norprotosinomenine has not previously been shown to be a precursor of the erythroidines (LI and LII).

R,S-N-Norprotosinomenine (XVII) was found to be 50 times a better precursor of erythraline (XXVI) than R,S-N-norreticuline (III) in E. crista galli. The possibility that S-N-norprotosinomenine (XXVII) is demethylated and subsequently remethylated to give S-N-norreticuline (I,R_1=R_2=H) which then follows the pathway (Scheme 1) proposed by Franck must be rejected in E. crista galli. The same argument also applies to E. berteroana (Table 4) where R,S-N-norprotosinomenine (XVII) was incorporated 7.5 times more efficiently than R,S-N-norreticuline (III) into the erythroidines (LI and LII).

The incorporation of R,S-N-norreticuline into the erythroidines in E. berteroana (0.008%) is larger than the incorporations into erythraline in E. crista galli of R-N-norprotosinomenine (XXVII enantiomer) (0.002% and 0.003%[4]) and bis-{2-(3-hydroxy-4-methoxyphenyl)} ethylamine (LIII) (0.0043% and 0.0012%[3]) which were considered insignificant.



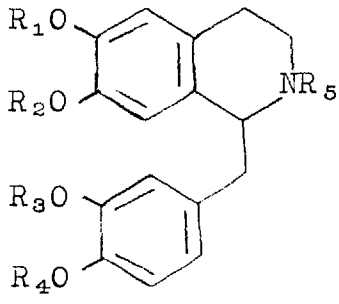
LIII

As R,S-[aryl-^{3}H]-N-norreticuline was present in the plant for 4 weeks 5 days it is not surprising that an incorporation which may be significant was obtained. Demethylation and remethylation of S-N-norreticuline (I, R_1=R_2=H, α-C_1-H) to give S-N-norprotosinomenine (XXVII) in E. berteroana has not been eliminated. There is also the very real possibility that over the long period of feeding, R,S-N-norreticuline has been degraded and the label 'scrambled'.

The possibility that a separate enzyme system exists in E. berteroana which directly oxidises N-norreticuline (Scheme 1) but less efficiently than the route via N-norprotosinomenine (Scheme 2, R_1=R_2=H) cannot be eliminated by this feeding result. In view of other work on the oxidative coupling of tetrahydrobenzyl -(and phenethyl-) isoquinolines in plant systems, a dual pathway to the same secondary metabolite is unlikely to be operating.

It has been well established that the correct methylation pattern of an oxygenated precursor is required in order that phenol oxidative coupling is directed in the required manner. The point is illustrated by work on Dicentra eximia[14]. Direct oxidative coupling of 1-benzyltetrahydroquinoline precursors to the aporphine alkaloids corydine (LIX) dicentrine (LXIII) and glaucine (LXIV) does not take place but instead, indirect coupling followed by a dienone-phenol rearrangement occurs. (Scheme 6). Acceptable incorporations were obtained[14] for tyrosine, 3-(3,4-dihydroxyphenyl) alanine, N-norlaudanosoline (LV), 4'-methyl-N-norlaudanosoline (LVI) and N-norprotosinomenine (XVII) into each aporphine alkaloid.

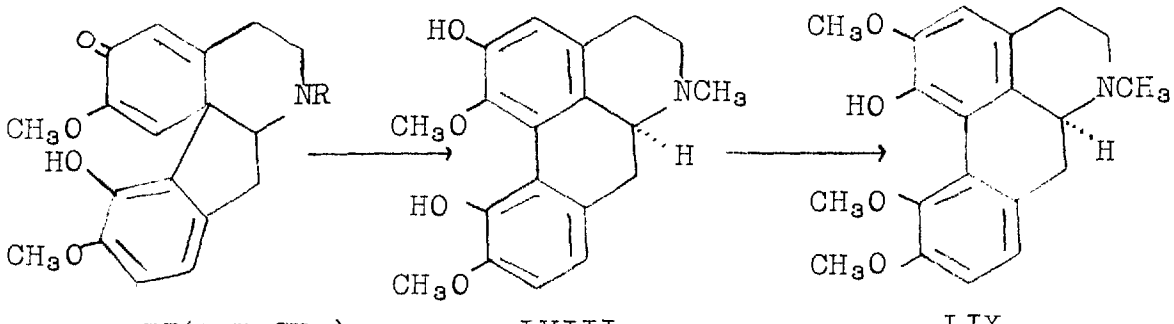
Scheme 6



LIV

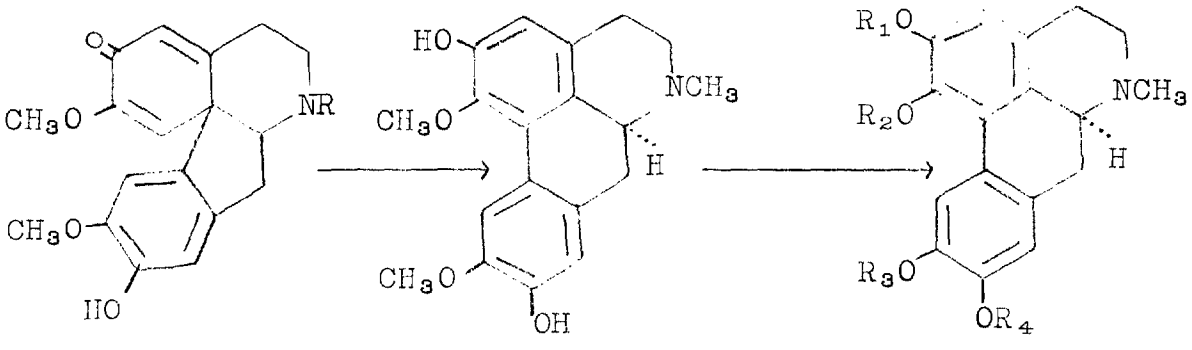
	R_1	R_2	R_3	R_4	R_5
LV	H	H	H	H	H
LVI	H	H	H	CH_3	H
XVII	H	CH_3	H	CH_3	H

LV ⟶ LVI ⟶ XVII ⟶



LVII(R=H,CH_3) LVIII LIX

⟶ OR



XXI LXI LXII

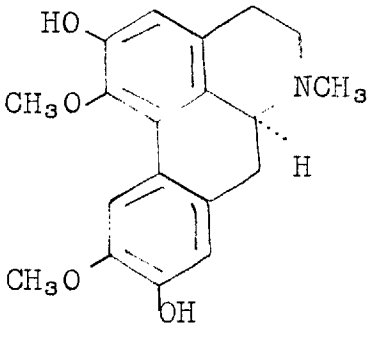
XXII R=H

LX R=CH_3

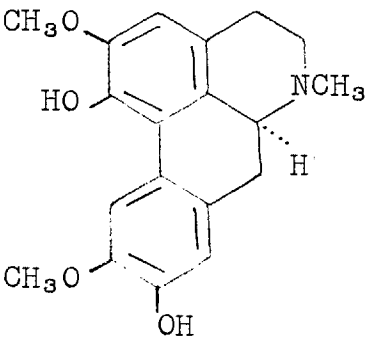
LXIII R_1,R_2=CH_2,R_3=R_4=CH_3

LXIV R_1=R_2=R_3=R_4=CH_3

Boldine (LXI) and not isoboldine (LXV) was incorporated into glaucine (LXIV) and dicentrine (LXIII) such that Scheme 6 was proposed



LXI



LXV

A large number of 1-benzylisoquinoline precursors with the 'wrong' O-and N-methylation patterns were fed and not incorporated (Table 5).

Table 5[14]

Benzylisoquinoline precursors not incorporated into aporphines in Dicentra eximia

R_1	R_2	R_3	R_4	R_5	(LIV, Scheme 6)
H	H	H	H	CH_3	laudanosoline
CH_3	H	H	H	H	
H	CH_3	H	H	H	
H	H	CH_3	H	H	
CH_3	H	H	CH_3	H	N-norreticuline (III)
CH_3	H	CH_3	H	H	N-nororientaline
CH_3	H	H	CH_3	CH_3	reticuline
CH_3	H	CH_3	H	CH_3	orientaline

2,11-Dimethoxy-10-hydroxy-6,7,7a,8-tetrahydroindano [1,2-j] isoquinoline-3(5H)-one (XXII) has been proposed as a precursor of the aporphine alkaloids[14] in Dicentra eximia (family Papaveraceae) and the Erythrina alkaloids in E.

crista galli[3] (Leguminoseae). It has been pointed out[14] that different families of plant use the same precursor in different ways (c.f. Schemes 2 and 6).

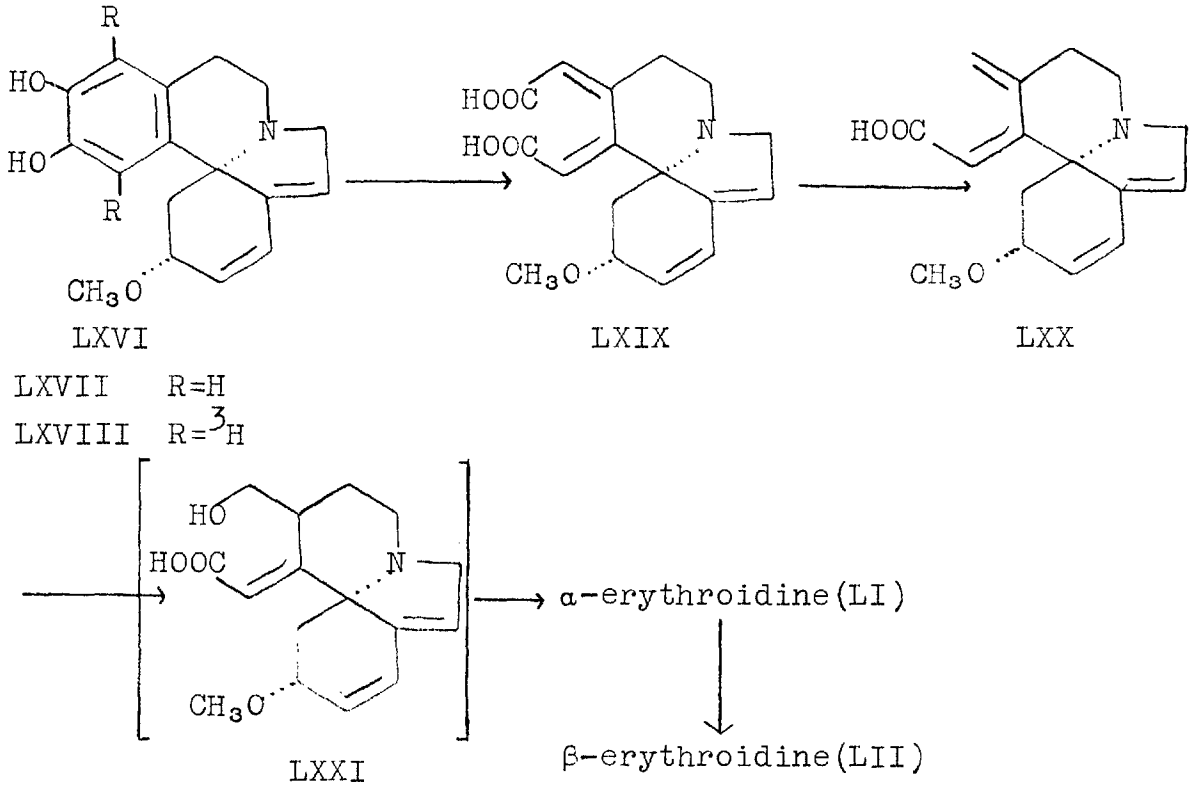
Similar examples of 1-benzyl-(and phenethyl-)isoquinolines which are correctly oxygenated but have an incorrect methylation pattern and are not incorporated, are to be found in Papaver orientale[15], P. dubium[16], P. somniferum[17,18,2], Croton linearis Jacq.[19] and Kreysigia multiflora[20] and Sinomenium acutum[31].

R-[Aryl-^{3}H]-N-nororientaline (XLVII) had effectively zero incorporation into erythraline in E. crista galli and the erythroidines in E. berteroana (Tables 2 and 4). A direct coupling pathway (Scheme 5) is not, therefore, operating in either Erythrina species. Naturally occurring R-N-nororientaline must either be formed by 'sideways' metabolism of R-N-norprotosinomenine or the methylation may have occurred some earlier stage in its own separate biosynthesis.

2. Feedings to the erythroidines in E. berteroana.

The structure and stereochemistry of α- and β-erythroidines (LI and LII) were elegantly deduced by Boekelheide[21]. R,S-[2-^{14}C]-Tyrosine had been shown to be incorporated (0.025%) specifically into positions 8 and 10 of the erythroidines in E. berteroana[12] and the pathway (Scheme 7) which was proposed[12,22] involved 'Woodward' fission[23] of the dihydroxy aromatic system of erysopine (LXVII). The intermediacy of erysopine (LXVII) or any other aromatic Erythrina alkaloid was not rigorously proved

Scheme 7[12,22]



LXVI

LXVII R=H

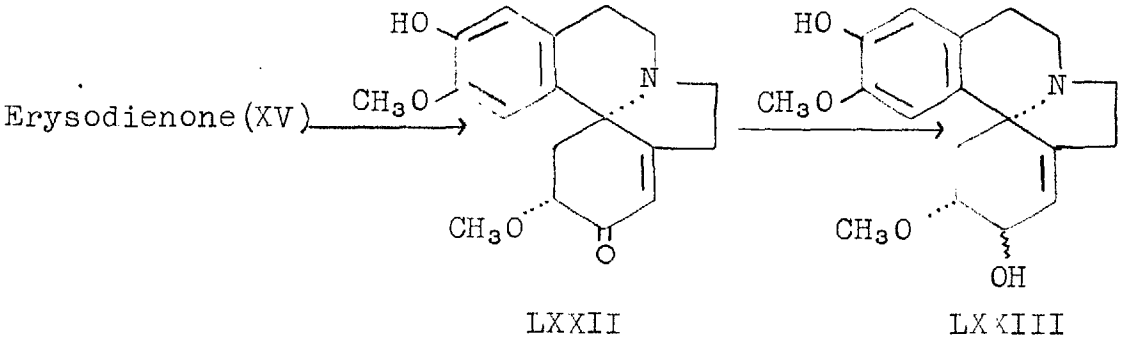
LXVIII R=^{3}H

LXIX

LXX

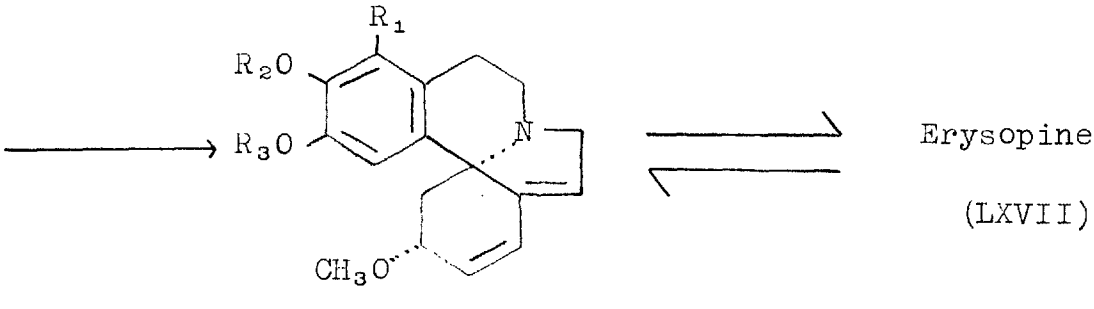
LXXI

Scheme 8



LXXII

LXXIII



LXXIV

LXXV R_1=H, R_2=H, R_3=CH_3

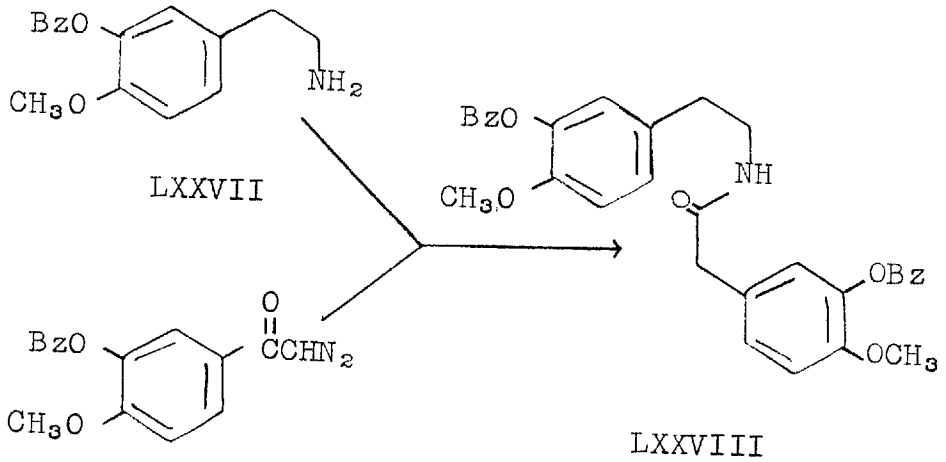
LXXVI R_1=^{3}H, R_2=H, R_3=CH_3

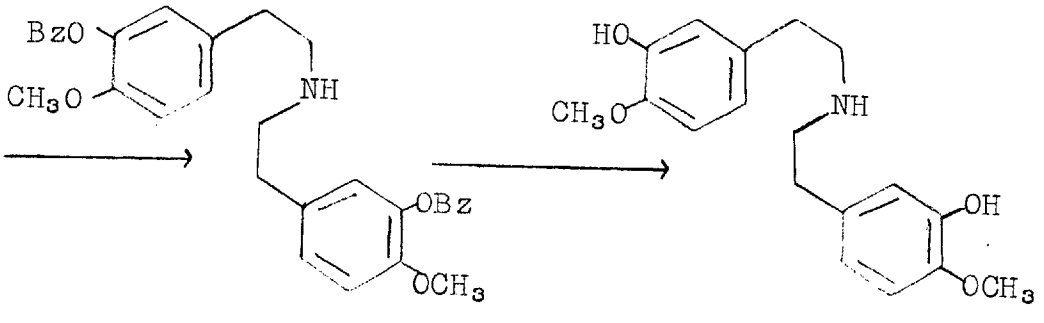
by feeding R,S-[2-^{14}C]-tyrosine. A pathway not involving erysodienone (XV) would be unlikely but any of the proposed intermediate compounds[24] between erysodienone and erysopine (Scheme 8) could be the specific precursor of the erythroidines.

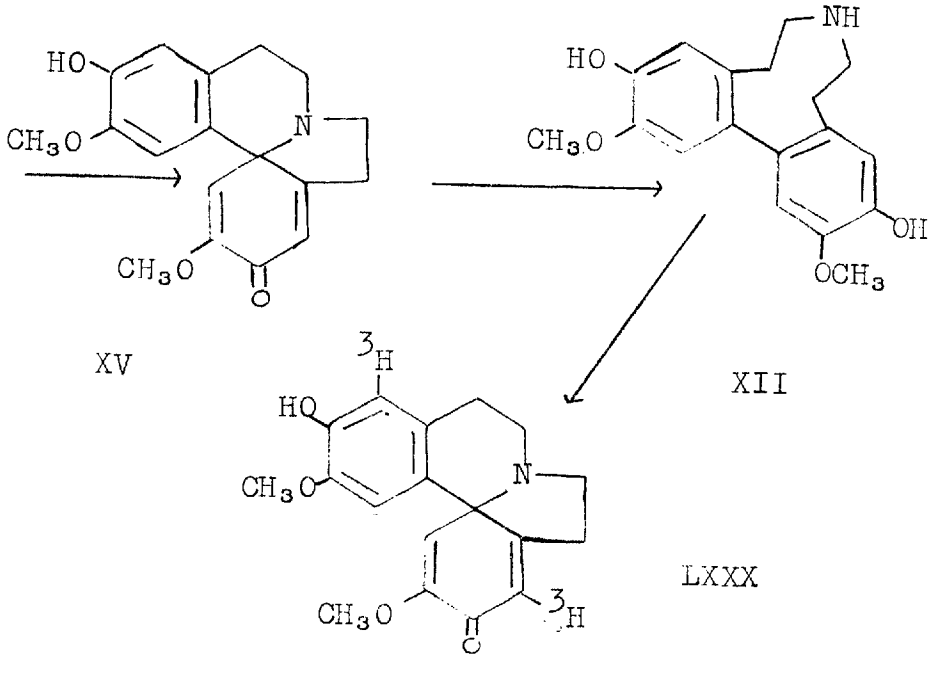
Erysodienone was synthesised by a modified route[3] (Scheme 9). 2-(3-Benzyloxy-4-methoxyphenyl)ethylamine (LXXVII) was photolysed[3] with 3-benzyloxy-4-methoxy-w-diazoacetophenone (XXXVIII) in benzene to give N-[2-(3-benzyloxy-4-methoxyphenyl)ethyl]-2-(3-benzyloxy-4-methoxyphenyl) acetamide (LXXVIII) in 56% yield. The amide was reduced with diborane[25] to bis-[2-(3-benzyloxy-4-methoxyphenyl) ethyl]amine (LXXIX) in 96% yield. Removal of the benzyl groups by hydrogenolysis over 10% palladium-carbon yielded bis-[2-(3-hydroxy-4-methoxyphenyl)ethyl]amine (95%) (LIII). The bisphenethylamine (LIII) was cyclised to R,S-erysodienone (XV)[25,26] in 30% yield using alkaline potassium ferricyanide in a two phase system (chloroform - water). Erysodienone was labelled by reducing it to R,S-5,6,8,9-tetrahydro-2,12-dimethoxy-7H-dibenz[d,f]azonine-3,11-diol (XII) with acidic chromous ion[3] and carrying out the usual base catalysed exchange with tritiated water. The label had previously[3] been shown by n.m.r. of the deuterated species to be incorporated in the 4 and 10 position of the tetrahydroazonine. Oxidation of the unpurified radioactive azonine in alkaline potassium ferricyanide gave R,S-[1,17-$^{3}H_2$]-erysodienone (LXXX) which was separated by t.l.c. and crystallised from ethanol.

Erysodine (LXXV) and erysopine (LXVII) were also labelled using base catalysed exchange with tritiated water.

Scheme 9


BzO
CH3O
NH2
LXXVII
O
CCHN2
XXXVIII
NH
OBz
OCH3
LXXVIII


BzO
CH3O
NH
OBz
OCH3
LXXIX
HO
OH
LIII


HO
CH3O
N
O
XV
NH
OH
OCH3
XII
3H
LXXX

Previous deuterium[4] exchange had shown that the compounds were [17-^{3}H]- erysodine (LXXVI) and [14,17-$^3H_2$]- erysopine (LXVIII).

The precursors were fed to 5 month old E. berteroana plants as aqueous solutions of their hydrochloride salts using the cotton wick method. For each precursor, two plants were used, one was harvested after 11 days and the other after 4 weeks 5 days. Each plant was worked up in the usual manner[3] and inactive α- erythroidine (LI) was added to the crude extract. Isolation of the mixture of α- and β - erythroidines (LI and LII) was carried out as above. The results are given in Tables 6 and 7. R,S - [2-^{14}C]- Tyrosine (L) and R,S-[5-^{3}H]- N-norprotosinomenine (XVII) were also fed but acceptable incorporations (0.032 and 0.060% respectively) were only obtained with the longer feedings experiment (Tables 3 and 4). R,S-[1,17-$^3H_2$]- Erysodienone (LXXX), [17-^{3}H]- erysodine (LXXVI) and [14,17-$^3H_2$]- erysopine (LXVIII) had satisfactory incorporations into the erythroidines in both sets of experiments using E. berteroana.

In order to confirm that the incorporation of the aromatic Erythrina alkaloids was specific, a suitable degradation of the erythroidines was elucidated (Scheme 10). Use was made of two previously known reactions, the isomerisation of α - erythroidine (LI) to β- erythroidine (LII)[27] with 10% sodium hydroxide solution and the reduction of β -erythroidine (LII) to β -erythroidinol[28] (LXXXV) by lithium aluminium hydride. The isomerisation was checked by comparison of the n.m.r. spectra of synthetic β -erythroidine and authentic

Table 6

Feedings to E. berteroana plants for 11 days.

Fed 26/5/72, harvested 6/6/72.

Precursor	Activity fed /10^4dps	Incorporation into erythroidines %
R,S-[1,17-$^3H_2$]-erysodienone(LXXX)	21.0	0.28±0.02
+-[17-^3H]-erysodine(LXXVI)	13.9	0.12±0.01
[14,17-$^3H_2$]-erysopine(LXVIII)	19.0	0.15±0.01

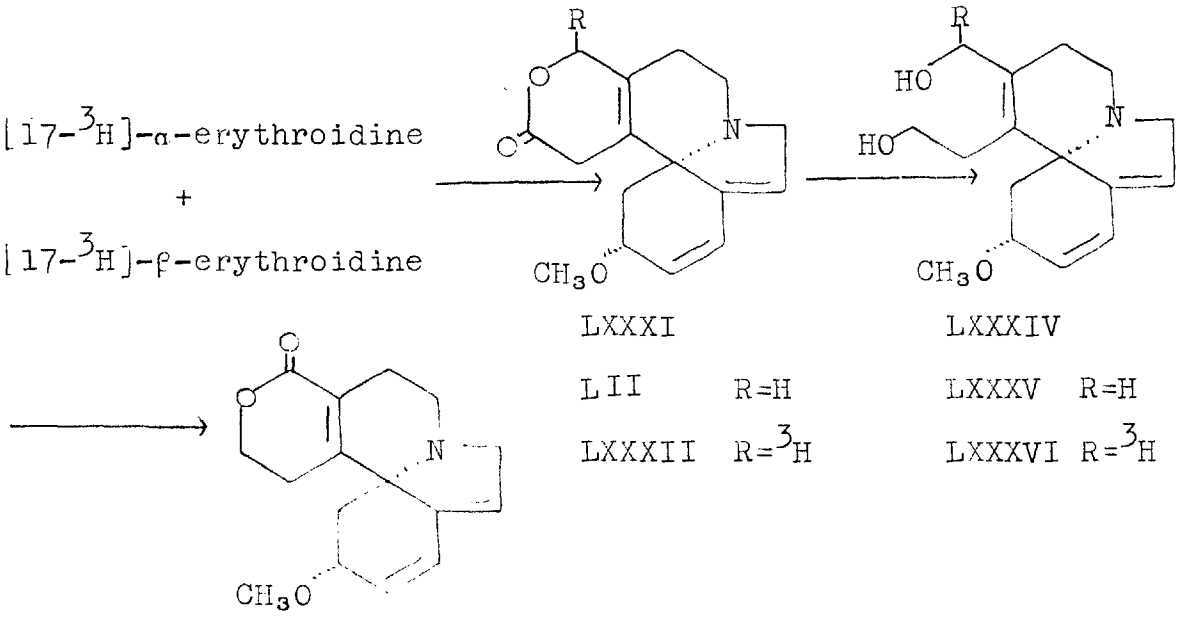
Table 7

Feedings to E. berteroana for 4 weeks 5 days.

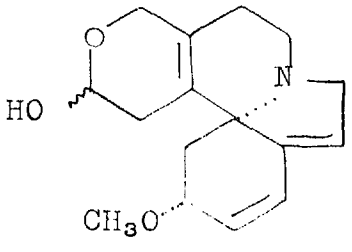
Fed 26/5/72, harvested 28/6/72.

Precursor	Activity fed /10^4dps	Incorporation into erythroidines %
R,S-[1,17-$^3H_2$]-erysodienone(LXXX)	28.0	0.078±0.006
+-[17-^3H]-erysodine(LXXVI)	14.0	0.50±0.04
[14,17-$^3H_2$]-erysopine(LXVIII)	18.1	0.21±0.03

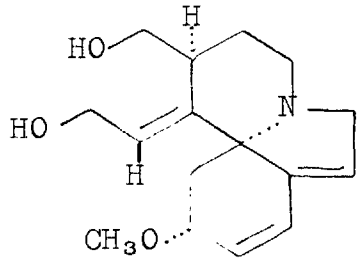
Scheme 10



material. They were completely superimposible showing no trace of the ABX multiplet ($J_{12,17A}$, 6Hz, $J_{12,17B}$, 9Hz, $J_{17A,17B}$, 12Hz) assigned to the 17 methylene group of ∝-erythroidine (LI). The literature[28] reduction of β- erythroidine using lithium aluminium hydride in ether at room temperature overnight gave a product showing two spots on t.l.c. The more polar spot was subsequently identified as β-erythroidinol (LXXXV) (M^+ 277). The less polar spot (M^+ 275, base peak 244) disappeared to give β-erythroidinol after refluxing and is presumed to be the intermediate hemiacetal (LXXXVIII). β-Erythroidine with lithium aluminium hydride in refluxing tetrahydrofuran gave β-erythroidinol (LXXXV) in 74% yield after 3 hours.



LXXXVIII



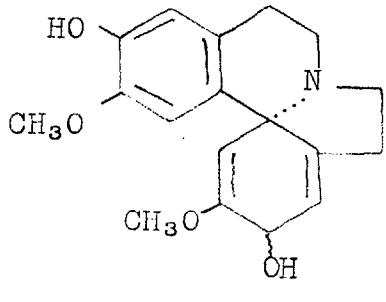
LXXXIX

The possibility that some of the isomeric α-erythroidinol (LXXXIX) would be formed during the reduction, was eliminated when α-erythroidine (LI) was reduced to α-erythroidinol (LXXXIX) in dry ether with lithium aluminium hydride[29]. Separation on t.l.c. gave α-erythroidinol in 50% yield. A signal in the n.m.r. at τ4.62 (t, $J_{14,15}$ 7Hz) corresponding to the olefinic 14 proton was not present in the n.m.r. spectrum of β-erythroidinol.

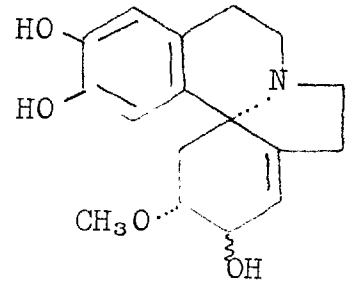
Oxidation of β-erythroidinol with manganese dioxide and silver carbonate on celite[30] gave more than four products according to t.l.c. but 15-desoxy-17-oxo- $\Delta^{12,13}$-erythroidine (LXXXVII) was obtained in 25% yield using a dilute solution of chromium trioxide in sulphuric acid. The lactone (LXXXVII) was crystallised as its hydrochloride monohydrate salt (m.p. 190-193° decomp.) and the water of crystallisation was not removed by drying at 60° *in vacuo* for 2.5 days.

Active erythroidine hydrochloride (45.1 dps mg^{-1}) from the feeding of [17-^{3}H]-erysodine (LXXVI) to *E. berteroana* for 4 weeks 5 days (Table 7, R=^{3}H) was diluted with inactive α-erythroidine (LI) and the sequence of reactions outlined above (Scheme 10, R=^{3}H) was performed. The resulting 15-desoxy-17-oxo- $\Delta^{12,13}$-erythroidine was crystallised as its hydrochloride monohydrate salt and found to have only 1% of the activity remaining i.e. 99± 6% of the activity had been removed. This is what would be expected for specific incorporation of [17-^{3}H]-erysodine into the erythroidines. The 17-^{3}H in [1,17-$^3H_2$]-erysodienone will be transferred to the erythroidines untouched if the above experiment and the incorporation of [1,17-$^3H_2$, methyl-^{14}C]-erysodienone (XV)[4] into erythraline (XXVI) in *E. crista galli* with no change in $^3H/^{14}C$ ratio are taken into account. The fate of 1-^{3}H of [1,17-^{3}H]-erysodienone and 14-^{3}H of [14,17-$^3H_2$]-erysopine cannot be accounted for, but most reasonable mechanisms suggest that they would not be lost or scrambled. Similarly the label in R,S-[5-^{3}H]-N-norprotosinomenine (XLIII) becomes, as confirmed by previous[4] double labelling experiments, the 17-^{3}H in the aromatic *Erythrina* alkaloidal skeleton.

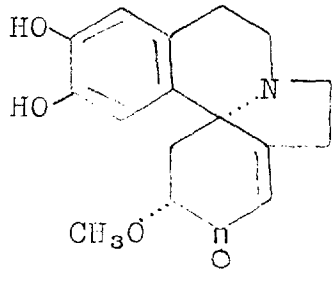
The good incorporations of erysodine and erysopine into the erythroidines in E. berteroana suggest but do not prove that erysopine is the precursor to the erythroidines. The direct pathway (Scheme 8) from erysodienone (XV) to erysodine (LXXV) is proposed[4,24] to involve reduction to erysotinone (LXXII), which may or may not proceed via erysodienols XC. Further reduction gives either or both of the epimers of erysotine (LXXIII) and elimination of the elements of water gives erysodine. Erysotinone (LXXII) and erysotine (LXXIII) had incorporations[4] of 0.31 and 0.18% respectively into erythraline in E. crista galli although the point of formation of the methylenedioxy group is not known. In order to supply a 1,2-dihydroxy system on which oxidative ring cleavage can occur, demethylation of erysodine (LXXV) to erysopine (LXVII) must take place. These results, however, do not preclude a pathway to the erythroidines via 15-desmethylerysotine (XCI), 15-desmethylerysotinone (XCII) or 15-desmethylerysodienone (XCIII).



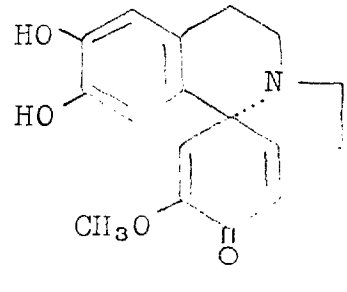
XC



XCI



XCII



XCIII

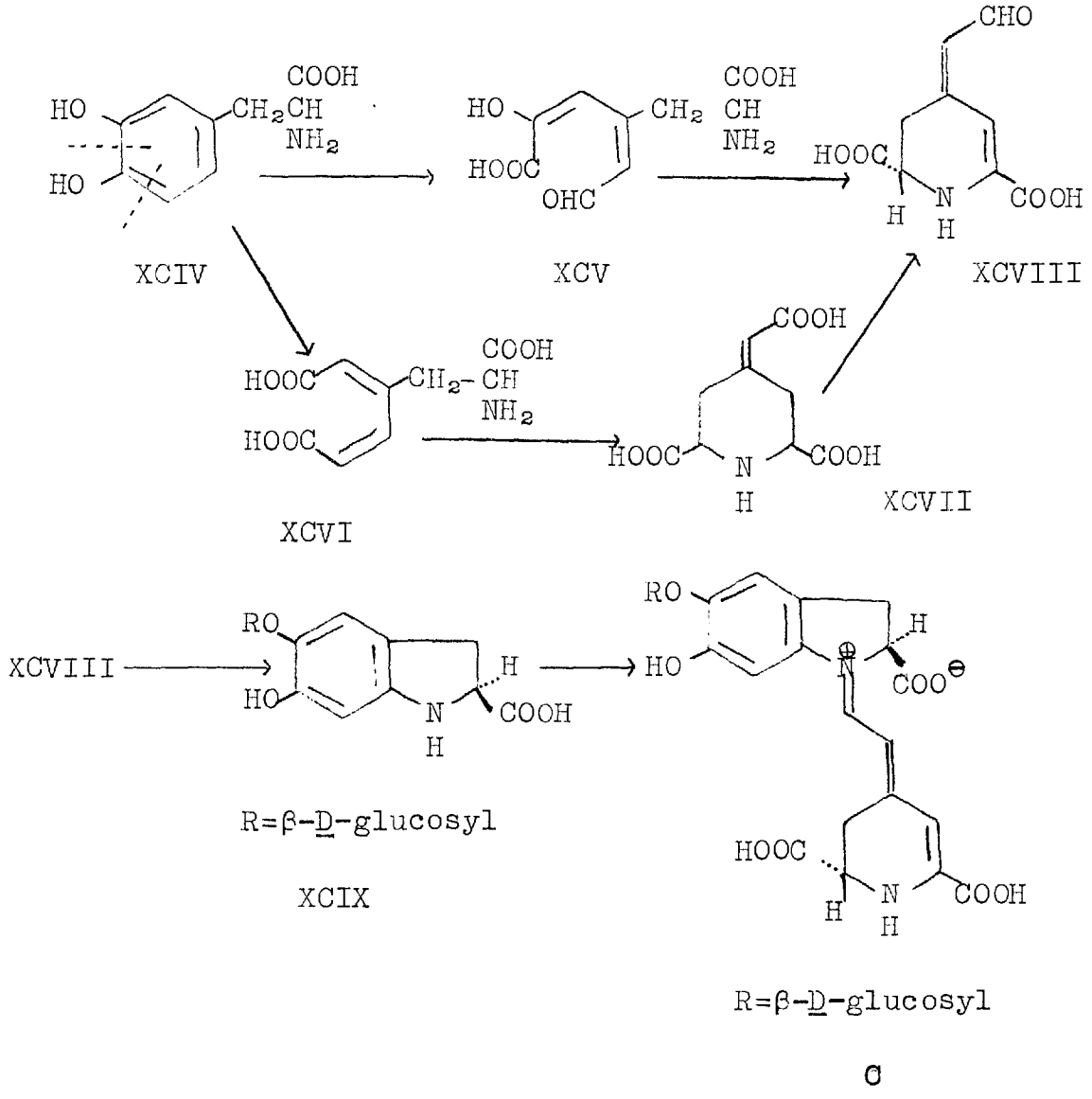
The incorporation of R,S-[5-^{3}H]-N-norprotosinomenine (XLIII) confirms that the formation of the aromatic Erythrina alkaloids in E. berteroana follows the pathway which has been unambiguously established in E. crista galli (Schemes 2 and 8)[3,4].

These experiments do not give any indication of the mechanism of the aromatic ring cleavage. In bacterial and fungal organisms, aromatic ring fission has been well studied but analogies in the plant kingdom are few. Dreiding and Mabry[32,33] have shown that R,S-3-(3,4-dihydroxyphenyl)-[1-^{14}C]-alanine (XCIV)([1-^{14}C]-DOPA) and [2-^{14}C]-DOPA are unambiguously incorporated into the tetrahydropyridine unit of betanin (C) in cactus fruits (Opuntia decumbens and O. bergeriana) (Scheme 11). These experiments did not allow them to predict the exact position of ring cleavage or its mechanism. The two possibilities, extradiol distal cleavage to give muconic semialdehyde XCV or intradiol fission to yield muconic acid XCVI were both considered.

Senoh proposed that S-stizolobinic(CIII) and S-stizolobic (CI) acids which were isolated from Stizolobium species[34,35] were biogenetically derived from S- DOPA (Scheme 12, R=S-3-alanyl). He also showed that 3,4-dihydroxyphenylacetic acid 2,3-dioxygenase, an enzyme which cleaves 3,4-dihydroxyphenylacetic acid (XCIV^{a},R=CH_2 COOH) between the 2 and 3 positions of the aromatic ring (B) to give the muconic semialdehyde (CII, R=CH_2 COOH), could also cleave DOPA in this position[36].

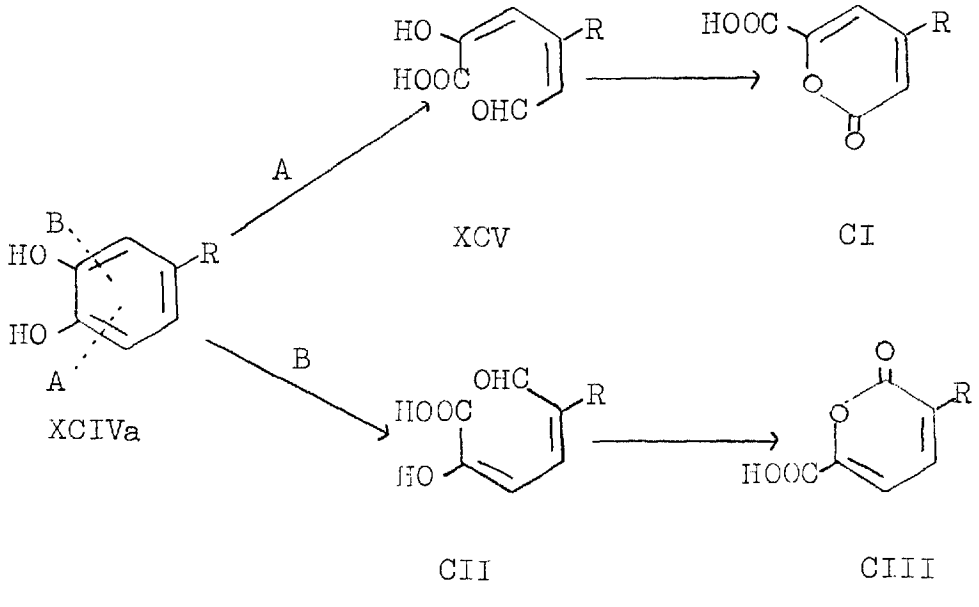
In view of the work of Senoh[36] and later Finkle[37], it has been suggested[36,38] that the enzymes called dioxygenases which have been well studied in and isolated from micro-

Scheme 11

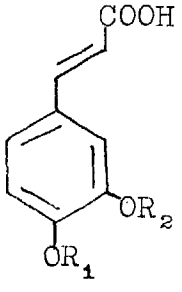


C

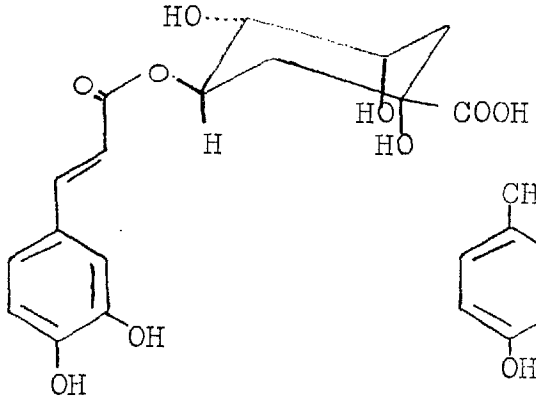
Scheme 12 (R=S-3-alanyl)



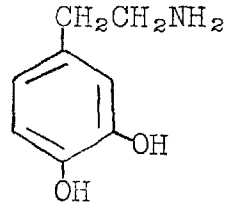
organisms are responsible for catalysing the cleavage of aromatic rings in plants. These enzymes act upon substrates which have a 1,2- or 1,4- dihydroxy aromatic ring system and molecular oxygen is necessary for the cleavage to occur[38]. By analogy to the Erythrina alkaloid oxygenation pattern, the 1,2-hydroxy system will only be considered. Finkle showed that caffeic acid (CV), chlorogenic acid (CVIII) and 2-(3,4-dihydroxyphenyl)ethylamine (CIX) reacted with protocatechuic acid 3,4-dioxygenase (intradiol) and metapyrocatechase (extradiol) enzyme preparations in the presence of oxygen. Ferulic (CVI) and isoferulic (CVII) acids were unaffected.



CIV



CVIII



CIX

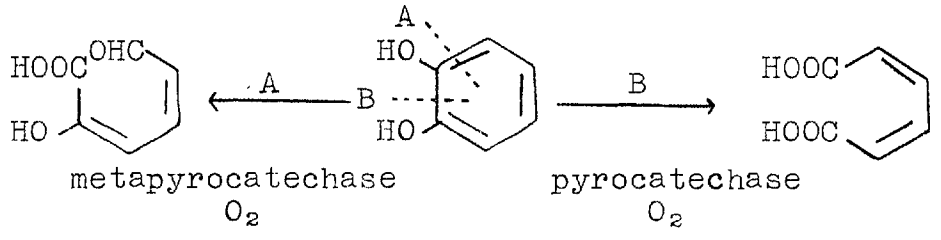
CV $R_1=R_2=H$

CVI $R_1=H, R_2=CH_3$

CVII $R_1=CH_3, R_2=H$

The simplest example of the 1,2-dihydroxy aromatic system is catechol (CX) which gives cis, cis-muconic acid (CXII) with the enzyme pyrocatechase and is cleaved to muconic semialdehyde (CXI) by the enzyme metapyrocatechase (Scheme 13)[38].

Scheme 13



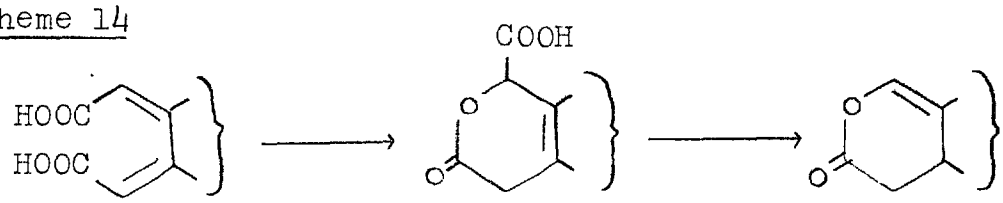
CXI CX CXII

Pyrocatechase has been obtained in a highly purified state from Pseudomonas arvilla[39] and metapyrocatechase has been isolated and crystallised[40] from P. arvilla. A whole range of compounds have been proposed as substrates for aromatic ring cleavage in bacteria and fungi (Review, Table 5). It has been observed[41] that intradiol cleavage only occurs with monocyclic aromatic substrates but this is not evidence that intradiol cleavage should be neglected for the aromatic Erythrina alkaloids.

As a result of the work summarised above it is proposed that a dioxygenase type enzyme is responsible for catalysing the aromatic ring cleavage in E. berteroana. The position of cleavage relative to the 1,2-dihydroxy aromatic unit of Erythrina alkaloids cannot be predicted from the available evidence. Pathways which have previously been proposed[12,22] (Scheme 7) involved what is now called intradiol cleavage and are still considered possibilities. A number of other possibilities from the diacid (LXIX) to the erythroidines can be considered. Among these are a) carboxylactone formation, b) hydration before decarboxylation. The diacid (CXIII) may lactonise and the resulting carboxylactone (CXIV) decarboxylate to give $\Delta^{12,17}$-erythroidine (CXV).

Double bond somerisation gives β - then α - erythroidine (Scheme 14).

Scheme 14

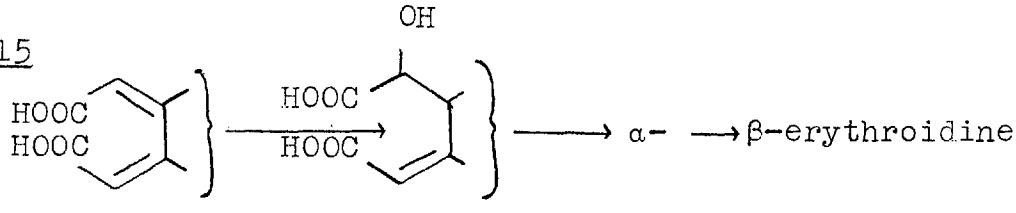


CXIII CXIV CXV

→ β-erythroidine (LII)

Alternatively, hydration of the diacid (CXIII) gives an α - hydroxy acid (CXVI) and decarboxylation will yield α - then β- erythroidine (Scheme 15).

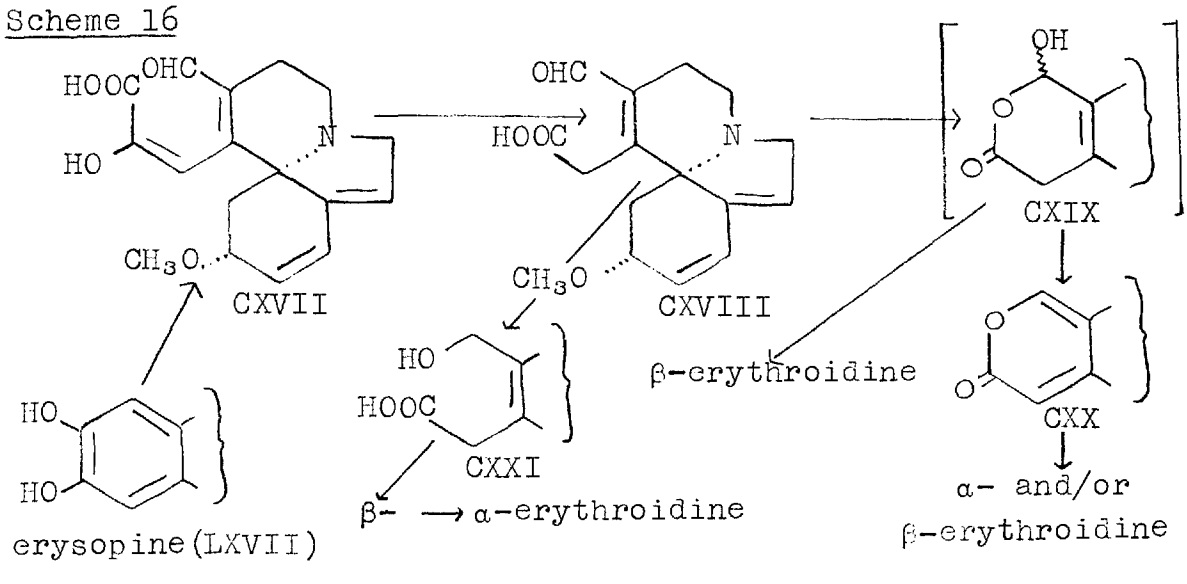
Scheme 15



CXIII CXVI LI LII

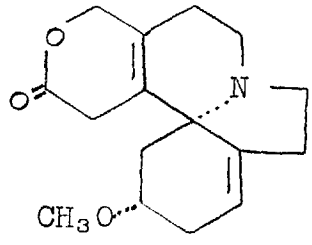
In addition extradiol cleavage must now be considered. If erysopine is the intermediate precursor then the product would be the muconic semialdehyde derivative CXVII (Scheme 16). Oxidative decarboxylation gives an aldo acid CXVIII and a series of relatively trivial steps yield α/β - erythroidine.

Scheme 16

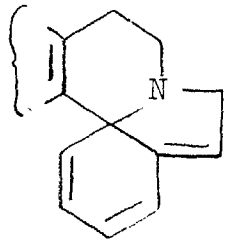


Isolation of the plant enzyme system would be a method of solving the problem of the mechanism and direction of ring cleavage in Erythrina species. The classical work of synthesising the two possible products of ring cleavage may also give some insight into the position of cleavage.

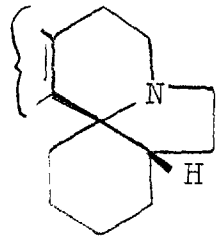
All the above possible pathways by which erysopine (LXVII) and erysodine (LXXV) are converted to the erythroidines do not involve any change on the stereochemistry at C-5. The incorporation of (+)-erysodine (LXXV) into the erythroidines in E. berteroana is further convincing evidence that the stereochemistry at C-3 and C-5 is the same in both the aromatic and lactone Erythrina alkaloids. The relative stereochemistry at C-3 and 5 of the lactone alkaloids was given by X-ray crystallographic analysis[42] of dihydro- β-erythroidine (CXXII) as cis. Degradation to a known 3-methoxyadipic acid[43] gave the absolute configuration at C-3 and hence C-5. The relative configuration, however, of the aromatic alkaloids has been deduced by X-ray analysis. Erythraline hydrobromide (XXVI) was shown[44] to have cis C_5-N and C_3-O. The absolute configuration of the aromatic alkaloids has been inferred[45] by molecular rotation difference of the desmethoxy (CXXIII) and hexahydrodesmethoxy (CXXIV) derivatives of the aromatic and lactone alkaloids. Also there was a close correlation of the o.r.d. of derivatives of the two classes but over the range of wavelengths chosen there was no Cotton effect[45]. An unambiguous chemical correlation of the two structures was unsuccessful[43].



CXXII



CXXIII



CXXIV

3. Alkaloid Metabolism in E. berteroana.

Throughout the 1971 season, various suspected precursors were fed to E. berteroana plants of differing degrees of maturity (Experimental Table). The wick feed method which proved successful in the 1972 season was also used to introduce the precursor into the plants. For first year plants (4-6 months old), incorporation into the erythroidines was not obtained when R,S-[2-^{14}C]-tyrosine (for 5 weeks 4 days) and R,S-[1,17-$^{3}H_2$]-erysodienone (LXXX) (for 7 days) were fed in mid-July. In mid-August, (±)-5,6,8,9-tetrahydro-2,12-[methyl-^{14}C]- dimethoxy-7H-[4,10-$^{3}H_2$]-dibenz[d,f]azonine-3,11-diol (XII) and R,S-[1,17-$^{3}H_2$]-erysodienone were fed for over 4 weeks and again no incorporation was observed. It is concluded, therefore, that first year plants can only be fed at the end of May and left into June. For mature plants (2nd year or above), incorporations into the erythroidines were obtained with R,S-[2-^{14}C]-tyrosine (0.013%) in a third year plant (feeding for 7 weeks 1 day), [17-^{3}H]-erysodine (0.067%) in a third year plant (6 days) and [14,17-$^{3}H_2$]-erysopine (0.12%) in a fourth year plant (11 days). These feedings were started in late May, late May and mid-June respectively. In parallel with the erysopine feeding, however, [17-^{3}H]-erysodine was refed to a fourth year plant

for 11 days and in this case there was no incorporation. One other feeding of [14,17-$^3H_2$]-erysopine to a second year plant in mid-August for 4 weeks 1 day was also 'negative'. It would, therefore, appear that incorporations can only be obtained using mature plants if they are fed at the end of May or early June but the plants are not as reliable as in the first year.

These results seem to indicate that the erythroidines are only being metabolised in the relatively short period starting towards the end of May and finishing in mid-June. Since the precursors were being introduced to whole plants via a cotton wick, it is possible that the transport mechanism which carries the precursor to the site of metabolism during early June, is for some unexplained reason not operating later in the season.

From the incorporations into the erythroidines in E. berteroana that were observed with first year plants (Tables 3,4,6 and 7), one of the four steps between N-norprotosinomenine (XVIII) and erysodienone (XV) (Scheme 2) must be slow or there is difficulty in transporting tyrosine and N-norprotosinomenine to their respective sites of secondary metabolism. Acceptable incorporations were obtained for erysodienone, erysodine and erysopine into the erythroidines after feeding for 11 days but tyrosine and N-norprotosinomenine were not incorporated in parallel experiments. A slow or inoperative step would appear to be more likely since if such a complicated structure as erysodienone is capable of reaching its metabolic site then enough N-norprotosinomenine ought to reach its site as well.

4. Other feeding methods with Erythrina species.

If it were possible to obtain incorporations into the erythroidines in germinating E. berteroana seedlings, then the restriction on feeding times would be eliminated. Successful incorporations into secondary metabolites in germinating or young seedlings have been obtained[46,47,48] in other plant species. A number of experiments were performed by adding the precursor (R,S-[2-^{14}C]-tyrosine, or [1,17-$^{3}H_2$]-erysodienone) to the medium in which seedlings were growing. In one experiment the roots were split to facilitate entry of the precursor into the seedling but in all cases, no activity was found in the isolated erythroidines. A similar experiment using E. crista galli seedlings was performed but, likewise, there was no activity in the isolated erythraline (XXVI).

The above results can be interpreted in four ways - a) there is no metabolism of Erythrina alkaloids during the first month of their growth, b) the precursor is not absorbed into the seedling, c) the precursor if it is absorbed, is not being transported to the site of metabolism d) the precursor is diluted in the medium such that not sufficient is incorporated.

An experiment was designed to eliminate the possibilities b) and d). One seedling of both the E. crista galli and the E. berteroana species was grown in a small polytop containing c.a. 1ml of R,S-[2-^{14}C]-tyrosine solution. The root of each seedling had been severed under water and kept throughout under the surface of water then precursor solution. Both

seedlings were left for 6 days before the whole seedling was sacrificed. Work up in the usual way gave erysodine (LXXV) and erythroidines (LI and LII) respectively, and both were found to be inactive. An aliquot of the remaining precursor solution was counted and it was found that 95% of the activity was absorbed into the E. berteroana seedling and 88% into the E. crista galli seedling. Considering the small size of the seedling (c.a. 8cm.), it must be concluded that the aromatic Erythrina alkaloids are not being metabolised in this period of their growth. The E. berteroana seedling was wilting after 6 days so by comparison with the length of feeding that is required in more mature plants, 6 days may not have been a long enough period of time for incorporation to take place. Previous work with E. crista galli indicated that 6 days is sufficient time for tyrosine to be incorporated into erythraline[3].

Cell free enzyme preparations have been widely used in the study of secondary metabolism especially in the fields of fungal, bacterial and mammalian metabolites but the technique has only had limited application with plants[49,50,51]. Two experiments were performed, one using E. berteroana seeds and the other with E. berteroana seedlings similar to those experiments that were performed with King Alfred daffodils[48] and pea seedlings[50]. The seeds or seedlings (12 days old) were crushed at -70° and the residue was shaken with a phosphate buffer and precursor solution at room temperature overnight. In neither case was there any incorporation into the extracted erythroidines. The technique as it was performed is not satisfactory for determining

the biosynthesis of the lactone Erythrina alkaloids.

5. Variation of alkaloid content with time in E. crista galli seedlings.

Because there was no incorporation of R,S-[2-^{14}C]-tyrosine into erythraline (XXVI) in E. crista galli seedlings, an investigation which could confirm the lack of alkaloid metabolism was initiated. For a given time interval, two E. crista galli seeds were germinated and grown and all were harvested at the same time. The work up of the two seedlings was performed in the usual manner to give a crude basic fraction which corresponded to the following ages of seedling - 0 (seeds), 1 week 3 days, 2 weeks 3 days, 4 weeks and 2 months. Approximately equal amounts of each extract were spotted on large t.l.c. plates and run in four solvent systems of increasing polarity against relevant authentic samples.

The percentage of total crude alkaloid was high in the seeds (2.1%) and fell sharply to c.a. 0.2% in the two month old seedlings. Erythraline (XXVI), erysodine (LXXV) and/or the isomeric erysovine (LXXIV, R_1=R_3=H, R_2=CH_3) were observed by co- t.l.c. Although there was a change in the percentage of crude alkaloid, the absolute amount of each of the above aromatic Erythrina alkaloids did not vary within the accuracy of the experiment. Erysotrine (LXXIV R_1=H, R_2=R_3=CH_3) which had not previously been isolated from E. crista galli, was identified and found to decrease in amount with the age of the seedling. An unknown polar alkaloid (R_f 0.4, alumina-5% methanol - chloroform) was not present in the seed or 2 month seedling extracts but was present in the other three.

Furthermore, this alkaloid was at a maximum after 2 weeks 3 days growth.

Erythraline was isolated by preparative t.l.c. and crystallised as its hydrobromide salt. Erysotrine (LXXIV R_1=H, R_2=R_3=CH_3) has previously been synthesised by methylation of erysodine[52] and has more recently been isolated from E. suberosa[53], E. lithosperma[10,54], E. aborescens[55], E. variegata[56], E. indica[56], and E. fusca[10]. It was isolated by preparative t.l.c. and had an identical mass spectrum to the literature[53]. The mass ion was precisely measured at 313:1684 which corresponds to $C_{19}H_{23}NO_3$. It also ran identically on t.l.c. with the product of reaction between diazomethane and erysodine (LXXV).

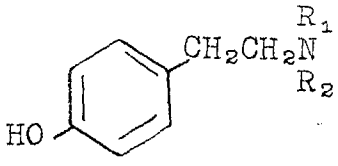
Erysodine/erysovine were only identified by co-t.l.c. with authentic materials and by the mass spectrum. Both isomers are identical in each of these tests.

The experiment was repeated using a single seedling for each time interval and it was again observed that an unknown alkaloid was present at a maximum after 2 weeks 2 days but not found in the extracts of the seeds or of a 1 month 13 day seedling.

The total amount of erythraline per plant does not appear to vary over the period of growth that has been investigated. Erythraline may be in a state of dynamic equilibrium with as much being formed as is being metabolised further but in view of the feeding results this would appear not to be the case. Erythraline, which is present in the seeds is, therefore, untouched for a certain period of the plants growth from seed until some point before the plant is 5 months

old when erythraline must be metabolised since R,S - [2-^{14}C]-tyrosine is then incorporated[3]. The biosynthesis of erysodine was also attempted with a feeding of R,S-[2-^{14}C]-tyrosine to an E. crista galli seedling and no incorporation was obtained. This is compatible with the above results when the total amount erysodine/erysovine was discovered to be unchanged during this early period of plant growth.

Some biosynthetic activity must occur in the plants since the unknown alkaloid varied in concentration from a seed to a one month old seedling. Such behaviour is known in other plant families e.g. Gramineae. In Hordeum species (barley), an early biogenetic study was made[57] into hordenine (CXXVI) after it was discovered that hordenine was not present in barley seeds, was a maximum in 11-17 day old seedlings and had disappeared after 30 days growth. Tyramine[58] (CXXVII) and N-methyltyramine[59] (CXXVIII) have also been shown to parallel this behaviour and it has been observed that the enzyme[59], tyramine methylpherase which regulates the levels of the above alkaloids, also appeared and disappeared in the same fashion. Other studies have shown that the alkaloid pattern in a number of plant families varies with age[57,58,59,60,61], degree of maturity[62,63], time of day[64,65] and environment[66]. Notably, the opium alkaloids from Papaver somniferum have been shown to have a varying alkaloid pattern over a 24 hour period[65] and the appearance and disappearance of codeine (CXXIX) and thebaine (CXXX) has been observed in the seedlings[48].

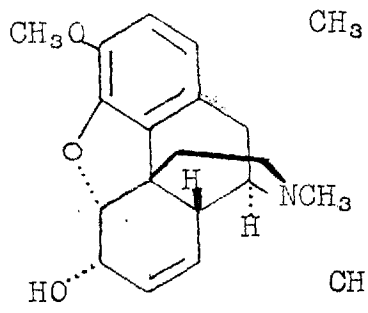


CXXV

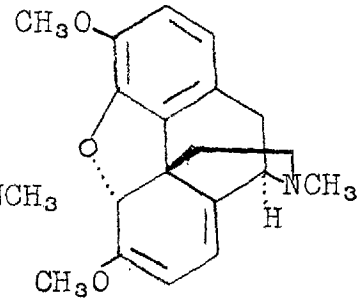
CXXVI $R_1=R_2=CH_3$

CXXVII $R_1=R_2=H$

CXXVIII $R_1=CH_3, R_2=H$



CXXIX



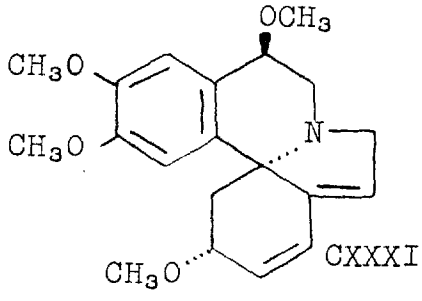
CXXX

6. Isolation and Partial Structure of the unknown alkaloid.

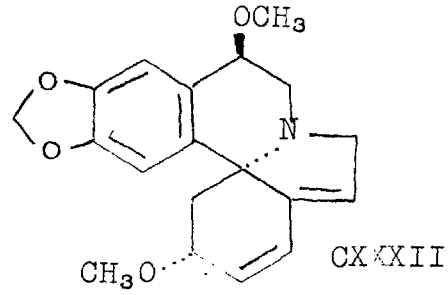
Two attempts were made to isolate and characterise the unknown alkaloid (R_f 0.4, alumina, 5% methanol-chloroform) from 2 week 3 day old E. crista galli seedlings. Extraction in the normal manner gave the crude alkaloidal fraction which was separated on t.l.c. to give the unknown alkaloid. Unfortunately, although this compound was always observed to be a single spot on t.l.c. even after being run three times, it could not be crystallised. The hydrochloride and picrate salts came out of solution as gums and the hydrochloride salt if left to dry became a glassy solid. This single spot may well be a mixture.

All the data are consistent with there being at least one, and possibly two, 1,6-diene aromatic Erythrina alkaloids present. The hydrochloride (glassy solid) had λ_{max} 236 ($E^{1\%}_{1cm}$500) and 287 nm (164) and although not elaborate the U.V. spectrum was similar to that of erythristemenine (CXXXI) (λ_{max} 283 (3100), 235(20,000)n.m.)[67]. The i.r.

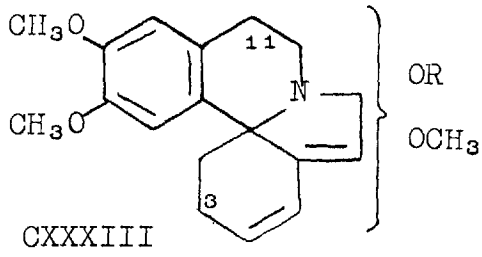
spectrum of the hydrochloride indicated only the presence of a hydroxyl (s) group (ν_{max} 3400 cm^{-1}) which was not phenolic since there was neither a shift in λ_{max} on the addition of base not was there any colour with ferric chloride solution.



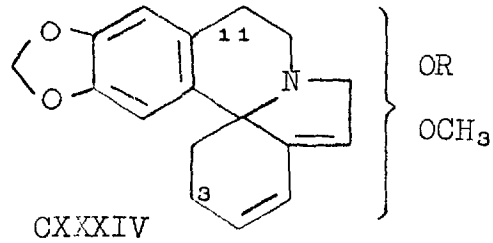
CXXXI



CXXXII



CXXXIII



CXXXIV

The mass spectra of the Erythrina alkaloids have been well studied[68] and using thesedata it is proposed that the isolated product is a mixture of at least two major compounds. Partial structures (CXXXIII and CXXXIV) can be tentatively written which account for all the significant peaks in the mass spectrum. R has not been identified but is probably not H since the isolated product was unaffected by prolonged treatment with active manganese dioxide. The usual oxygenation pattern of Erythrina alkaloids would place the hydroxyl at an allylic 3 or 11 position. A plausible fragmentation pattern for both structures and comparison with observed spectra has been set out in Table 8. The mass spectrum was very similar to that of erythristemine (CXXXI)[67] and 11-methoxy-erythraline[69] (CXXXII). For both structures CXXXIII and CXXXIV the same fragmentation pattern is used to produce

Table 8

Comparison of the observed fragmentation pattern and that proposed for structures CXXXIII and CXXXIV.

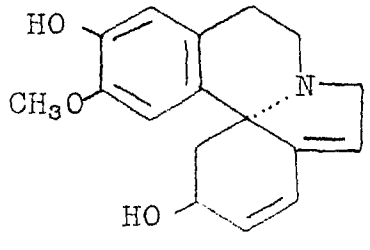
Observed peaks/m/e	Proposed peaks/ m/e CXXXIII			CXXXIV		
329	329		-R, +H			
313 $C_{19}H_{23}NO_3$ and $C_{18}H_{19}NO_4$	313	$C_{19}H_{23}NO_3$	-OR, +H	313	$C_{18}H_{19}NO_4$	-R, +H
311 $C_{19}H_{21}NO_3$	311	$C_{19}H_{21}NO_3$	-OR, -H			
298 $C_{18}H_{20}NO_3$	298	$C_{18}H_{20}NO_3$	-OR, $-CH_3$, +H			
297 $C_{18}H_{19}NO_3$				297	$C_{18}H_{19}NO_3$	-OR, +H
282 $C_{18}H_{20}NO_2$ and $C_{17}H_{16}NO_3$	282	$C_{18}H_{20}NO_2$	-OR, $-OCH_3$ +H	282	$C_{17}H_{16}NO_3$	-OR, $-CH_3$ +H
280 $C_{18}H_{18}NO_2$ and $C_{17}H_{14}NO_3$	280	$C_{18}H_{18}NO_2$	-HOR, $-OCH_3$	280	$C_{17}H_{14}NO_3$	-HOR, $-CH_3$
266 $C_{17}H_{16}NO_2$				266	$C_{17}H_{16}NO_2$	-OR, +H, $-OCH_3$
264 $C_{17}H_{14}NO_2$				264	$C_{17}H_{14}NO_2$	-HOR, $-OCH_3$

Table 9

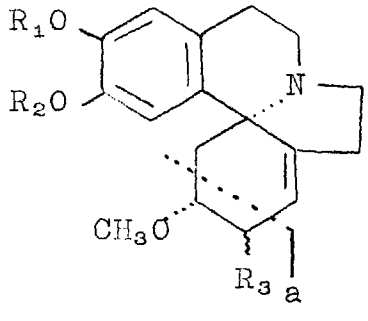
Possibilities for the base peak from a compound with a 1,(6) double bond.

	Structure CXXXVII	+'O'	+'OCH_3'
$R_1=R_2=H$	229	245	259
$R_1,R_2=H,R_2,R_1=CH_3$	243	259	273
$R_1,R_2=CH_2$	241	257	271
$R_1=R_2=CH_3$	257	273	287

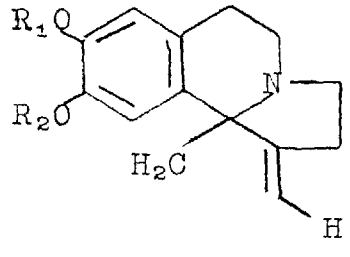
the observed peaks. Further evidence for R≠H can be gained from the observation of the m/e 329 and 313($C_{18}H_{19}NO_4$) peaks which would become mass ions but their relative intensities to the base peak (14% and 17%) are very small for these systems. For comparison, erysonine (CXXXV) was prepared[70] by dilute acid hydrolysis of erysodine (LXXV) and it only had peaks in the mass spectrum at m/e 285(100%, M^+) and 268. The erysodienols (XC, all possibilities) were prepared by sodium borohydride reduction of erysodienone (XV) and they had peaks in the mass spectrum at m/e 315(60%, M^+) and 298 (100%). Evidence that a dienol system was not present could be inferred from the lack of an enol ether peak (1650 cm^{-1}) in the i.r. spectrum of the isolated product.



CXXXV



CXXXVI



CXXXVII

Confirmation of the lack of a 1(6) double bond (e.g. as in erythratine (CXXXVI, R_1,R_2=CH_2, R_3=OH) can also be obtained from the mass spectrum of the isolated product. The base peak from 1(6) double bond compounds[68] arises by cleavage indicated by 'a' (CXXXVI). In table 9, the masses are given for the ions (CXXXVII) with all possible groups R_1 and R_2 and for added oxygenation and O-methylation in

that part of the molecule. In the observed spectrum, the peaks given in Table 9 had no significance, moreover, there were no large peaks in positions ± 2 mass units from those in Table 9.

The n.m.r. spectrum of the isolated product could not confirm the partial structures (CXXXIII and CXXXIV) but was not in disagreement with them. Since the product is thought to be a mixture, the integration is not valid. The presence of aromatic protons was shown by signals at τ 3.30-3.70 (complex). There were four complex signals at τ 3.87, 4.00, 4.14 and 4.29 beneath which could be the methylenedioxy signal. In 11-methoxyerythraline[69] the O-CH_2-O signal is found at 4.67 but in 11-hydroxyerythraline[71] the signal is quoted as having a chemical shift of τ4.06. There are 3-singlets at τ6.21, 6.32 and 6.72 assigned to O-CH_3 with the signal at τ6.72 agreeing well with other 3 methoxy assignments[67,69,71].

Acetylation of a small sample on which hydrochloride salt formation had been attempted, gave a product which had a mass spectrum consistent with the structures (CXXXIII R=CH_3CO- and CXXIV R=CH_3CO) being present. Peaks at m/e 371 (M^+), 356 (M^+-CH_3) 340 (M^+-OCH_3) can be assigned to (CXXXIII R=CH_3CO-) and the peaks at 355 (M^+), 340 (M^+-CH_3) and 324 (M^+-OCH_3) correspond to the other acetylated structure (CXXXIV R=CH_3CO). If R≠H in the original structures of the isolated product then hydrolysis must have taken place in the presence of the more concentrated acid which is found when wet ethanolic hydrogen chloride is used. The structures (CXXXIII R=H) and (CXXXIV R=H)

are then acetylated.

In order to obtain the full structure and stereochemistry of the unknown product, more E. crista galli seeds are necessary which can be germinated and grown for the relevant period of time. Controlled hydrolytic or reductive removal of the R group is necessary, such that the alkaloidal parts of the structure can be determined. Aromatic Erythrina alkaloids have been isolated as their carboxymethylsulphonic acid esters[72] and as glycosides[73].

Conclusion

The work reported in this thesis has confirmed that, in common with other plant families, Erythrina species require the correct hydroxylation pattern of 1-benzyl-tetrahydroisoquinoline precursors in order for the correct phenol oxidative step to occur and produce the Erythrina alkaloids. The biogenetic scheme which was proposed by Franck[1] and had N-norreticuline as an intermediate was shown to have no significance in E. crista galli.

The aromatic Erythrina alkaloids have been firmly established as precursors of the erythroidines in E. berteroana. The successful feeding to E. berteroana plants by the wick feed method can only be accomplished at the end of May or in early June.

During the first two months of a seedlings growth from seed it appears that metabolism of erythraline and erysodine does not occur in E. crista galli. An unidentified alkaloid which was not present in the seed or 1 month old seedling extracts is present in relatively high concentrations after 2 weeks 3 days.

EXPERIMENTAL

Melting points were determined on a Kofler hot stage apparatus. N.m.r. refer to deuterochloroform solutions containing about two percent tetramethylsilane as an internal standard and were recorded on either a Varian T60 spectrometer or a Varian HA100 spectrometer. Unless otherwise stated infrared spectra refer to chloroform and ultra violet spectra to absolute ethanol solutions. Mass spectra were obtained with an A.E.I. MS9 double-focussing instrument; samples were introduced by direct probe insertion with an ionisation potential of 70ev. Column chromatography was on neutral alumina of Brockman activity III and thin layer chromatography (t.l.c.) on plates prepared using either Merck Aluminium Oxide $G.F._{254}$ or Silica Gel $G.F._{254}$. Light petroleum is the fraction boiling between 60° and 80°.

Unless otherwise stated t.l.c. was performed using alumina plates.

Preparation of labelled Precursors.

Counting of Radioactive Compounds - Radioactive assays were made, unless otherwise indicated, on a Nuclear Enterprises Ltd. N.E. 8310 Liquid Scintillation Counter using a solution of either the active compound (ca. 0.5 m.g.) from a feeding or a sample (10 µl) of a solution of more active precursor [ca. 0.5 m.g. in methanol (10ml)], in analar methanol (2ml) and Nuclear Enterprises Ltd. type 240 scintillation liquid (8ml). Internal standardisation with [1-^{14}C]-n-hexadecane and [1,2-$^{3}H_2$]-n-hexadecane allowed the counting efficiency to be determined for each sample.

A Beckmann Liquid Scintillation Counter (courtesy of Imperial College Biochemistry Department) was also used with 2-methoxyethanol (3ml) and Scintillator Liquid (10ml) as solvent. The machine had an internal standard and was regularly calibrated. Low background counting phials were used with both machines.

Base catalysed exchange with tritium oxide[3] - The phenolic bases were dissolved in redistilled N,N-dimethylacetamide - tritium oxide, sealed under nitrogen and kept at 118° for 3 days. The solvents were evaporated in vacuo and the phenols were either crystallised from suitable solvents or separated on t.l.c. and isolated as crystalline free bases or hydrochloride salts. N.m.r. of the deuterium oxide exchanged phenol gave the position of the label.

R,S-[2-^{14}C]-tyrosine (L). This was obtained from the Radiochemical Centre, Amersham. The sample (50µC) was dissolved in 0.01M hydrochloric acid (10ml) and this solution (2µl) counted.

The activity was $1.92 \pm 0.04 \times 10^5$ dps. ml^{-1}.

N-[2-(4-Benzyloxy-3-methoxyphenyl)ethyl]-2-(3-benzyloxy-4-methoxyphenyl)acetamide (XXXIX)[8] - 2-(4-Benzyloxy-3-methoxyphenyl)ethylamine (XXXV) (740mg)[7] and 3-benzyloxy-4-methoxy-ω-diazoacetophenone[8] (XXXVIII) (960mg) in dry benzene (250ml) were irradiated, without cooling and under an atmosphere of nitrogen, with a 125W. high pressure mercury vapour lamp for 3hr. (i.r. control). The solution was washed with 0.2M - hydrochloric acid (2 x 50ml), 1M - sodium hydroxide solution (50ml) and water (50ml), dried (Na_2SO_4) and evaporated. The amide crystallised from chloroform/light petroleum (900mg., 61%) m.p. 140-142° (lit.[8] 139-141°) and had $\nu_{max.}$ (nujol) 3310 and 1640 cm^{-1}.

7-Benzyloxy-1-(3-benzyloxy-4-methoxybenzyl)3,4-dihydro-6-methoxyisoquinoline hydrochloride (XL)[9] - N-[2-(4-Benzyloxy-3-methoxyphenyl)ethyl]2-(3-benzyloxy-4-methoxyphenyl)acetamide (XXXIX) (500mg) in dry toluene (8ml) was refluxed with phosphorusoxychloride (0.15ml) for 1hr. The solvents were removed in vacuo and a solution of hydrogen chloride in ethanol was added. The ethanol was evaporated to give a foam which gave the imine (XL) as its hydrochloride salt from methanol/ether (323mg., 62%), m.p. 198°-200° (lit.[74] 203-204°), $\nu_{max.}$ (nujol) 1655(w) and 1610(w); significant peaks in the mass spectrum occurred at m/e 493, 402 (base peak), 312,310, 269.

R,S-7-Benzyloxy-1-(3-benzyloxy-4-methoxybenzyl)-6-methoxy-1,2,3,4-tetrahydroisoquinoline (XLI) - To a stirred solution of the free imine base (XL) (420mg) in methanol (15ml), excess sodium borohydride (c.a. 1.0g) was added and the suspension

was stirred for 3 hours. The solvent was evaporated *in vacuo* and to the solid was added water (10ml) and 0.6*M* - hydrochloric acid until acidic. The solution was made basic with solid sodium hydrogencarbonate and extracted with chloroform (4 x 10ml). The chloroform solution was dried over Na_2SO_4 and evaporated under reduced pressure. The oil (382mg., 91%) was treated with a solution of hydrogen chloride in ethanol, to give the *O*,*O*-dibenzyl-*N*-norreticuline (XLI) as its hydrochloride salt (350mg., 76%) m.p. 150-152° (lit. 149-150.5°[75] and 165°[76]). The free base had major peaks in the mass spectrum at *m/e* 495, 402, 364, 282, 268, (base peak), 228, 227, 177, 176, 137, 91 and τ2.43-2.85 (broad m, 10H, aromatic - *H*), 3.18-3.50 (broad m, 5H, H- 2', 5', 6', 5,8), 4.81 and 4.93 (two s, 4H, O-CH_2), 6.05 (broad m, 1H, H-1), 6.17 (s, 6H, O-CH_3), 6.60-7.40 (broad m, 7H, CH_2 + N-*H*).

R,S-*7-Hydroxy-1-(3-hydroxy-4-methoxybenzyl)-6-methoxy-1,2,3,4-tetrahydroisoquinoline, (N-norreticuline)* (III) - *O*,*O*-Dibenzyl-*N*-norreticuline hydrochloride (XLI) (100mg) was refluxed under nitrogen in a mixture of ethanol (5ml) and concentrated hydrochloric acid (2.5ml) for 2 hours. Water (10ml) was added and the solution was made basic with solid sodium hydrogen carbonate and extracted with chloroform (4 x 10ml). The chloroform solution was dried over Na_2SO_4 and evaporated. The residue was treated with a solution of hydrogen chloride in ethanol and after removal of the solvent *in vacuo*, *N*-norreticuline hydrochloride (56mg., 85%) (III) was isolated as its monohydrate[18] from methanol/ether m.p. 160-163° (lit., monohydrate[18] 165-166°,

245°[76]); τ 3.26 (broad s, 3H, H-2',4',5'), 3.45 (broad s, 2H, H-5,8), 5.57 (broad m, 3H, O-H/N-H) 5.90 (broad m, 1H, H-1), 6.15 (s, 6H, O-CH_3), 6.7 - 7.5 (broad m, 6H, CH_2). Significant peaks in the mass spectrum were at m/e 315, 178 (base peak), 163, 137.

R,S -7-Hydroxy-1-(3-hydroxy-4-methoxy-[2,6-$^3H_2$]-benzyl)-6-methoxy-1,2,3,4-tetrahydro-[8-^3H]-isoquinoline (XLII) - Inactive N-norreticuline (III) (15mg) as its free base was exchanged with tritium oxide in the usual manner. The crude residue was separated on t.l.c. (5% chloroform/methanol) to give [aryl-^3H]-N-norreticuline hydrochloride monohydrate (XLII) (9mg., 51%). Exchange with deuterium oxide showed loss of -3 aromatic protons by n.m.r. The activity was $2.36 \pm 0.14 \times 10^5$ dps mg^{-1}.

R-7-Hydroxy-1-(4-hydroxy-3-methoxy-[5-^3H]-benzyl)-6-methoxy-1,2,3,4-tetrahydro-[8-^3H]-isoquinoline, (R-[aryl-^3H]-N-nororientaline) (XLVII) - R-N-Nororientaline (XLVI) was isolated from Erythrina poeppigiana, Walp. (Venezuela) by Miss A. Lobo[10] and the free base had mp. 144.5 - 146.5 (ethyl acetate), $[\alpha]_D^+$ 42 ($CHCl_3$), τ (D_2O) 3.20-3.43 (m, 5H, aromatic -H), 5.93 (m, 1H, H-1), 6.20 (s, 3H, O-CH_3), 6.23 (s, 3H, O-CH_3) 6.77-7.23 (complex, 6H,-CH_2-) and major peaks at m/e 315 (M^+), 178 (base peak), 163,137. This alkaloid exchanged two protons with deuterium when heated with deuterium oxide in presence of base. Base catalysed exchange with tritium oxide in the usual manner gave R-[aryl-^3H]-N-nororientaline (XLVII) isolated as its hydrochloride salt m.p. 247-249° from methanol-ether (lit.[71] mp. 249-50°) and had an activity of $7.70 \pm 0.4 \times 10^4$ dps. mg^{-1}.

R,S-[5-^{3}H]-N-Norprotosinomenine (XLIII) - This had previously been prepared from [2,6-$^3H_2$]-isovanillin[77] (XLIV). The hydrochloride salt which showed a single spot on t.l.c. (chloroform - 5% methanol) was recrystallised (methanol-ether) m.p. 240-242° (lit.[3] 241-240°). The activity was $2.45 \pm 0.10 \times 10^4$ dps mg.$^{-1}$.

(+)-[17-^{3}H]-Erysodine (LXXVI)[4]- Base catalysed exchange of inactive erysodine (14mg.) ($[\alpha]_D$ +225, lit.[78] + 231 ($CHCl_3$)) and recrystallisation from absolute ethanol gave (+)-[17-^{3}H]-erysodine (8.0mg., 57%) (LXXVI) m.p. 205-206° (lit.[78] 204-205°) with an activity of $7.7 \pm 0.5 \times 10^4$ dps. mg.$^{-1}$.

[14,17 - $^3H_2$]-Erysopine (LXVIII)[4] - Inactive erysopine (18.0mg.) gave [14,17-$^3H_2$]-erysopine (9.2mg., 51%) (LXVIII) m.p. 238° (dec.) (lit.[78] 241-242) from 95% ethanol. The activity was $1.13 \pm 0.06 \times 10^5$ dps mg.$^{-1}$.

R,S-[1,17 -$^3H_2$]-Erysodienone (LXXX) - Previously published work[3,4] was used as a basis for the synthesis (Scheme 9).

N-[2-(3-Benzyloxy-4-methoxyphenyl)ethyl]-2-(3-benzyloxy-4-methoxyphenyl) acetamide (LXXVIII) - The 3-benzyloxy-4-methoxy-w-diazoacetophenone[8] (XXXVIII) (9.0g) and 2-(3-benzyloxy-4-methoxyphenyl)ethylamine[31] (LXXVII) (8.0g) were irradiated in dry benzene (1.5l), under an atmosphere of nitrogen, with a 125W high pressure mercury lamp until the diazo absorption (2150cm^{-1}) in the i.r. spectrum had disappeared (21hr.). The solution was reduced *in vacuo* to smaller volume (c.a. 500ml.), washed with 0.6M- hydrochloric acid (50ml), 0.4M- sodium hydroxide solution (50ml) and water (2 x 50ml)., and dried over Na_2SO_4. Treatment of the hot solution with activated charcoal, filtration and further reduction in the volume gave

the amide (LXXVIII) (8.9g, 56%); m.p. and m.m.p. 114.5-116° (benzene) (lit. 113.5-115°) (MeOH)[79], $\nu_{max.}$ (nujol) 3330 and 1640cm^{-1}.

Bis-[2-(3-benzyloxy-4-methoxyphenyl)ethyl]amine Hydrochloride (LXXIX) - Diborane was produced by slowly adding a solution of boron trifluoride etherate (21.0g) in diglyme (50ml) dropwise to a stirred suspension of sodium borohydride (3.0g) in diglyme (50ml). The diborane was swept with a slow stream of nitrogen into a stirred solution of the amide (LXXVIII) (6.0g) in dry tetrahydrofuran (50ml) at room temperature. An acetone trap prevented diborane coming into contact with air. The stirred suspension was refluxed for 2hr., dilute hydrochloric acid (80ml) added and refluxed for a further 3hr. (until white spot corresponding to diborane complex on t.l.c. had disappeared). The tetrahydrofuran was evaporated off from the solution under reduced pressure and the aqueous solution was made basic with solid sodium hydrogen carbonate, extracted with ether (3 x 100ml). The combined ether solutions were dried over Na_2SO_4 and the amine hydrochloride salt (LXXIX) (6.0g, 96%) m.p. 168-170° (ethanol-ether)[80]. crystallised after adding HCl gas.

Bis-[2-(3-hydroxy-4-methoxyphenyl)ethyl]amine Hydrochloride (LIII) - The O,O-dibenzylamine hydrochloride (LXXIX) (534mg) was partially dissolved in absolute ethanol (150ml) containing 3 drops of concentrated hydrochloric acid. The amine was hydrogenated at atmospheric pressure and ambient temperature using 10% palladium on charcoal (20mg)as catalyst until completion (50ml). The suspension was heated to boiling, filtered and the catalyst washed with more hot

ethanol. The combined filtrates were evaporated to small bulk and cooled to give the phenolic amine hydrochloride (LIII) (336mg, 95%) m.p. 230-232° (lit[3]. 228-233° dec. from N,N dimethyl formamide-di-isopropyl ether).

R,S- Erysodienone[3,25,26] (XV) - A solution of potassium ferricyanide (3.52g) in 5% sodium carbonate solution (400ml) was added dropwise at room temperature to a stirred solution of the phenolic amine hydrochloride (LIII) (921mg) in chloroform (3l) under an atmosphere of nitrogen over a period of 15 minutes. The chloroform layer was separated and the aqueous phase washed with chloroform (3 x 200ml). The combined chloroform extracts were washed with water (400ml), dried (Na_2SO_4) and evaporated under reduced pressure. Separation on t.l.c. (5% methanol-chloroform) gave erysodienone (XV) (246mg, 30%) m.p. 218-220° (dec) (lit.[3] 226-230°) (from ethanol).

R,S-5,6,8,9-Tetrahydro-2,12-dimethoxy-7H-dibenz[d,f]azonine-3,11-diol[3] (XII) - Erysodienone (XV) (51.0mg) was stirred with a 1M solution of chromous chloride in 3% hydrochloric acid (7.5ml) under an atmosphere of nitrogen for 45 min. at room temperature. Air was allowed to enter the system and the pH was adjusted to 8 with solid sodium hydrogencarbonate. The solid was filtered off and washed with hot ethanol (3 x 50ml). The filtrate was extracted with chloroform (3 x 20ml) and the combined ethanol and chloroform extracts were evaporated under reduced pressure, redissolved in chloroform (50ml), dried (Na_2SO_4) and evaporated under reduced pressure. The biphenyl (XII) (37.8mg., 74%) was recrystallised from absolute ethanol m.p. 222-224° (lit.[3] m.p. 222-224°) and

showed no presence of erysodienone (XV) in the i.r. or on t.l.c. (5% methanol-chloroform or acetone-silica).

(±)-5,6,8,9-Tetrahydro-2,12-dimethoxy-7H-[4,10-$^3H_2$]-dibenz[d,f]azonine-3,11 diol (XII)[4]- The usual base - catalysed exchange of the biphenyl (XII) with tritium oxide and evaporation of the solvents gave a foamy solid which was not purified at this stage.

R,S-[1,17-$^3H_2$]-Erysodienone (LXXX)[4] - The radioactive tetrahydroazonine (XII) (37.8mg) in chloroform (150ml) was added dropwise to a stirred solution of 5% sodium hydrogen carbonate (10ml) containing potassium ferricyanide (60ml) under an atmosphere of nitrogen at room temperature. After 45min. stirring, the chloroform layer was removed and the aqueous layer extracted with chloroform (3 x 20ml). The combined chloroform solutions were dried (Na_2SO_4) and evaporated under reduced pressure to give a gum which was separated on t.l.c. (acetone-silica) to give R,S-[1,17-$^3H_2$]-erysodienone (LXXX) (12.0mg., 31%) from ethanol (twice), m.p. 219-220° (dec.), with an activity of 1.18 ± 0.06 x 10^5 d.p.s. mg^{-1}.

Feedings to Erythrina crista galli in 1972 Season.

General Method[81]- The precursor (c.a. 2mg., 10μC.) as its hydrochloride salt was dissolved in water (1.0ml) and the solution was divided equally between two small polytops which were cello-taped to the stem of a healthy first-year (5month old) E. crista galli plant. Into each polytop a piece of untreated cotton was dipping which had been threaded efficiently through the stem of the plant two or three times. Watering of the plant was stopped and the precursor allowed to pass via the cotton into the plant. The flask which contained the precursor solution was washed with distilled water and the washings used to transfer any precursor left in the polytops or on the cotton, into the plant. Watering was continued after four days or when the plant started wilting, whichever came sooner, and the plant harvested after 11 days, a longer period than in previous years because the weather was poor. Two plants were used for each experiment.

For each of the four precursors - R,S-[2-^{14}C]-tyrosine (L), R,S-[aryl-^{3}H]-N-norreticuline (XLII), R-[aryl-^{3}H]-N-nororientaline (XLVII) and R,S-[5-^{3}H]-N-norprotosinomenine (XLIII), two plants germinated in February 1972 were used. The experiments were started on 26th May 1972 and all the plants were harvested on 6th June 1972.

Work up - The two whole plants were macerated in a Waring Blender with 0.06M - hydrochloric acid (300ml) and then stirred at room temperature for 6 days. The suspension was left under an atmosphere of nitrogen in the cold room until work up could be continued.

The suspension was filtered through celite and the filtrate washed once with light petroleum (50ml). Inactive erythraline hydrobromide (XXVI) (c.a. 14mg) was added and the solution after it was basified with solid sodium hydrogencarbonate, was extracted with chloroform (6 x 50ml). The combined chloroform extracts were dried (Na_2SO_4) and evaporated under reduced pressure to give a gum. Separation by t.l.c. (50% ethyl acetate-benzene) gave erythraline (XXVI) which was recrystallised as its hydrobromide salt twice from ethanol before any radioactive counting was attempted.

Extraction of inactive erythraline (XXVI) - Old *E. crista galli* plants (2 years and older) (600g) were macerated and stirred with 0.06M - hydrochloric acid (2l.) for 3 days at room temperature. The extract was filtered through celite, extracted with chloroform (6 x 250ml) and the combined chloroform solutions dried (Na_2SO_4), evaporated under reduced pressure to give the crude alkaloids (380mg., 0.06%). Separation on a column (10% ethyl acetate-benzene) then on t.l.c. (50% ethyl acetate-benzene) gave erythraline (XXVI) as its hydrobromide salt (94mg) from ethanol m.p. 246-249° (dec.), $[\alpha]_D^{22}$ + 216° (c 1.0) (H_2O) [lit.[3] m.p. 240-243°, $[\alpha]_D^{25}$ +219° (H_2O)] and had peaks in the mass spectrum[68] at m/e 297 (M^+), 282 and 266 (base peak).

R,S-[2-^{14}C]-Tyrosine (L) to *E. crista galli*[3] - R,S-[2-^{14}C]-Tyrosine (L) (1ml, 1.92 ± 0.04 x 10^5 dps) was fed to two plants on 26/5/72 and harvested on 6/6/72 (wet weight 9.3g). It was diluted with erythraline hydrobromide (13.9mg) to give total crude alkaloid (27.9mg).

Activity of erythraline hydrobromide after 2 re-crystallisations 23.7 ± 0.9 dps mg^{-1} (Beckmann)

Activity of erythraline hydrobromide after 3 re-crystallisations 26.8 ± 1.0 dps mg^{-1} (Beckmann).

Incorporation = 0.18 ± 0.02%

R,S-[5-^{3}H]-N-Norprotosinomenine (XLIII) to E. crista galli.- R,S-[5-^{3}H]-N-Norprotosinomenine hydrochloride (XLIII) (2.08mg., 5.1 ± 0.2 x 10^4dps) was fed on 26/5/72 and harvested 6/6/72 (wet weight 10.8g). After dilution with inactive material (14.5mg), an activity of 1.80 ± 0.06 dps. mg^{-1} (after 2 re-crystallisations) and 1.74 ± 0.06 dps. mg^{-1} (after 3 re-crystallisations) was produced in the erythraline hydrobromide isolated. Erythraline was re-separated as its free base on t.l.c. (50% ethyl acetate-benzene) and again crystallised as its hydrobromide salt with a activity of 1.79 ± 0.05 dps. mg^{-1} (Beckmann).

Incorporation 0.051 ± 0.005%

R,S-[aryl-^{3}H]- N -Norreticuline to E. crista galli.

R,S-[Aryl-^{3}H]-N-norreticuline hydrochloride (XLII) (0.90mg., 2.10 ± 0.15 x 10^5 dps) was fed on 26/5/72 and harvested 6/6/72 (wet weight 11.02g). After dilution with inactive material (12.6mg), the activity of the isolated erythraline hydrobromide was 0.11 ± 0.02 dps. mg^{-1} (2 re-crystallisations).

Incorporation 6.5 ± 1.5 x 10^{-4}% (0.001% after allowing for loss of 1 tritium atom).

R-[aryl-^{3}H]-N-Nororientaline to E. crista galli.

R-[Aryl-^{3}H]-N-nororientaline hydrochloride (XLVII)

(2.90mg, 2.25 ± 0.10 x 10^5 dps) was fed on 26/5/72 and harvested on 6/6/72 (wet weight 7.3g). After dilution with inactive material (14.4mg) the activity of the isolated erythraline hydrobromide was 0.05 ± 0.02 dps mg^{-1} (3 re-crystallisations).

Incorporation less than 10^{-4}%

Feedings to Erythrina berteroana in 1972 Season.

General Method - The precursor (c.a. 2mg., 10μC) was dissolved as its free base in 0.006M - hydrochloric acid solution (1ml) or if a hydrochloride salt then it was dissolved in water (1ml). The solution was introduced into one small polytop attached to the E. berteroana plant (5 months old) which had some untreated cotton threaded twice through its stem. The cotton dipped into the precursor solution in the polytop. E. berteroana plants (10cm high,) which had been germinated in February 1972 from seeds kindly supplied by Tropical Products Institute were smaller and more fragile than E. crista galli plants (30cm high) of the same age. Watering of the plant was stopped and the precursor solution allowed to pass into the plant. The empty polytop was refilled with distilled water so that any remaining precursor was washed into the plant. Watering of a plant was restarted when it was considered that it was beginning to wilt. The plants were harvested in two batches, one batch 11 days after feeding and the other after 4 weeks 5 days.

Work up - The whole plant was macerated in a Waring Blender with 0.06M - hydrochloric acid (150ml) and stirred for 6 days at room temperature. The suspension was left in the cold room under an atmosphere of nitrogen until work up could be continued.

The suspension was filtered through celite and the filtrate washed once with light petroleum (25ml). Inactive α-erythroidine hydrochloride (LI) (c.a. 14.0mg) was added and the solution was basified with solid sodium hydrogen

carbonate and extracted with chloroform (6 x 30ml). The combined chloroform extracts were dried (Na_2SO_4) and evaporated under reduced pressure to give a gum. Separation by t.l.c. (50% ethyl acetate-benzene) gave $\alpha + \beta$ erythroidine mixture which was recrystallised[13] as its hydrochloride salt (methanol-ether) three times before any radioactive counting was attempted.

α-Erythroidine hydrochloride (LI) - Dr. K. Folkers and Messrs. Merck and Co., Rahway, New Jersey had kindly supplied α- erythroidine hydrochloride (LI). The free base was separated on t.l.c. (50% ethyl acetate-benzene) and crystallised as its hydrochloride salt m.p. 220-225° (dec.) (lit. m.p.[78] 228-229° dec.). The free base had τ 3.62 (d. of d., 1H, $J_{1,2}$ 10Hz, $J_{2,7}$ 2Hz, H-2), 4.14 (m, 1H, $J_{1,2}$ 10Hz, H-1), 4.27 (m, 2H, H-7 and H-14), 5.59 (m, 1H, $J_{17A/17B}$ 12Hz, $J_{12,17A}$ 6Hz, H-17A), 6.00 (m, 1H, $J_{17A,17B}$ 12Hz, $J_{12,17B}$ 9Hz, H-17B), 6.02 (broad m, 1H, H-3), 6.38 (d, 2H, J 2Hz, H-8), 6.63 (s, 3H, O-CH_3), 6.6-7.6 (complex, 4H, H-10, 11) 8.0-8.4 (complex, 3H, H-12, 4).

β-Erythroidine hydrochloride (LII) - Messrs. Merck and Co., also kindly supplied this alkaloid. The hydrochloride salt in methanol was heated with charcoal, filtered and some of the solvent removed under reduced pressure. Addition of dry ether produced β - erythroidine hydrochloride (LII) as needles m.p. 225-227° (dec.) (lit. m.p.[78] 232° dec.). The free base had τ 3.57 (d. of d., 1H, $J_{1,2}$ 10Hz, $J_{2,7}$ 2Hz H-2), 4.11 (m, 1H, $J_{1,2}$ 10Hz, H-1), 4.27 (m, 1H, $J_{1,7}$ 2Hz, H-7). 5.38 (m, 2H, H-17), 5.95 (complex, 1H, H-3), 6.42 (broad s, 2H, H-8), 6.62 (s, 3H, O-CH_3), 6.64-7.2 (complex, 4H, H-10, 14), 7.2-7.9 (complex, 2H, H-11), 8.0-8.5 (complex, 2H, H-4).

11 Day feedings to E. berteroana in 1972 (Tables 3 and 6)

R,S-[2-^{14}C]-Tyrosine to E. berteroana.

R,S-[2-^{14}C]-Tyrosine (1ml., 1.92 ± 0.04 x 10^5 dps) was fed to one plant on 26/5/72 and harvested on 6/6/72 (wet weight c.a. 3g). After dilution with inactive material (15.9mg), crude alkaloid (14.0mg) was produced from which weakly active erythroidine hydrochloride (0.30 ± 0.02 dps mg^{-1}, incorporation 2.5 ± 0.2 x 10^{-3}%) was separated.

R,S-[5-^{3}H]-N-Norprotosinomenine (XLIII) to E. berteroana.

The hydrochloride salt (2.20mg., 5.40 ± 0.20 x 10^4 dps) was fed to one plant on 26/5/72 and harvested on 6/6/72. After dilution with inactive material (14.6mg), crude alkaloid (13.3mg) was separated from which inactive erythroidine hydrochloride was isolated (incorporation less than 10^{-4}%).

As a consequence of the previous two results, the experiments feeding R,S-[aryl-^{3}H]-N-norreticuline hydrochloride (XLII) (1.07mg., 2.50 ± 0.15 x 10^5 dps) and R-[aryl-^{3}H]-N-nororientaline hydrochloride (XLVII) (3.10mg., 2.4 ± 0.1 x 10^5 dps) on 26/5/72 and harvesting on 6/6/72 to E. berteroana plants were not worked up.

R,S-[1,17-$^3H_2$]- Erysodienone (LXXX) to E. berteroana.

R,S-[1,17-^{3}H]-Erysodienone (LXXX) (1.78mg., 2.1 ± 0.1 x 10^5 dps) was fed to one plant as its hydrochloride salt on 26/5/72 and harvested on 6/6/72. Dilution with inactive α-erythroidine hydrochloride (15.0mg) gave crude alkaloid (18.6mg).

After separation the erythroidine hydrochloride had

Activity/dps mg^{-1}	Number of Recrystallisations
42.5 ± 2.5	4
37.0 ± 2.5	5
40.5 ± 2.5	6

Incorporation $\underline{\underline{0.28 \pm 0.02\%}}$.

[17-^{3}H]- Erysodine (LXXVI) to E. berteroana

[17-^{3}H]-Erysodine (LXXVI) (1.80mg., $1.39 \pm 0.07 \times 10^5$dps) was fed as its hydrochloride salt on 26/5/72 and harvested on 6/6/72. Inactive α- erythroidine hydrochloride (15.9mg) was added and gave crude alkaloid (18.4mg) from which active erythroidine as its hydrochloride salt was separated.

Activity/dps mg^{-1}	Number of Recrystallisations
9.6 ± 0.6	3
11.4 ± 0.8	4
10.4 ± 0.7	5

Incorporation = $\underline{\underline{0.12 \pm 0.01\%}}$

[14,17-$^3H_2$]- Erysopine (LXVIII) to E. berteroana.

[14,17-$^3H_2$]-Erysopine (LXVIII) (1.70 mg., $1.90 \pm 0.10 \times 10^5$ dps) was fed to one plant as its hydrochloride salt on 26/5/72 and harvested on 6/6/72. Inactive α -erythroidine hydrochloride (14.6mg) was added and gave crude alkaloid (15.9mg). Active erythroidine hydrochloride was separated.

Activity/dps mg^{-1}	Number of Recrystallisations
20.5 ± 1.0	3
19.0 ± 1.0	4
20.0 ± 1.0	5

Incorporation 0.15 ± 0.01%

4 weeks 5 days Feeding Experiments with Erythrina berteroana in 1972 (Tables 4 and 7).

R,S-[2-^{14}C]-Tyrosine (L) to E. berteroana.

The precursor (1ml., 1.92 ± 0.04 x 10^5 dps) was fed to one plant on 26/5/72 and harvested on 28/6/72 (wet weight 18.7g). Inactive α- erythroidine hydrochloride (14.6mg) was added and crude alkaloid (14.0mg) was separated. The erythroidine hydrochloride isolated was active.

Activity/dps mg^{-1} (Beckmann)	Number of recrystallisations
4.3 ± 0.2	3 (Beckmann)
4.1 ± 0.2	4 (")

Incorporation 0.032 ± 0.002%

R,S-[5-^{3}H]-N-Norprotosinomenine (XLIII) to E. berteroana.

The hydrochloride salt (2.05mg., 5.0 ± 0.2 x 10^4 dps) was fed on 26/5/72 and harvested on 28/6/72 (wet weight 3.0g). Inactive α- erythroidine hydrochloride (13.8mg) was added and crude alkaloid (11.7mg) was separated. Active erythroidine hydrochloride was isolated.

Activity/dps mg^{-1}	Number of Recrystallisations
2.30 ± 0.15	4
2.08 ± 0.15	5

The free base was run again on t.l.c. (50% ethyl acetate-benzene) to give erythroidine hydrochloride (2.16 dps mg^{-1}).

Incorporation 0.060 ± 0.005%

R,S-[aryl-^{3}H]-N-Norreticuline (XLII) to E. berteroana.

The hydrochloride salt (1.08mg., 2.55 ± 0.15 x 10^5 dps) was fed to one plant on 26/5/72 and worked up on 28/6/72 (wet weight 2.65g). Inactive α-erythroidine hydrochloride (15.9mg)

was added and crude alkaloid (13.8mg) was isolated. The isolated erythroidine was counted as its hydrochloride salt.

Activity/dps mg^{-1}	Number of recrystallisations
0.91 ± 0.05	3
0.94 ± 0.05	4
0.90 ± 0.05	5

Incorporation 0.0056 ± 0.004% (0.008% allowing for loss of 1 tritium).

R- [aryl-^{3}H]-N-Nororientaline (XLVII) to E. berteroana.

The precursor (XLVII) as its hydrochloride salt (3.22 mg., 2.50 ± 0.10 x 10^5 dps) was fed to one plant on 26/5/72 and harvested on 28/6/72 (2.12g plant weight). Inactive α-erythroidine hydrochloride (15.5mg) was added and crude alkaloid (15.8mg) was separated. The isolated erythroidine hydrochloride had effectively zero activity after 4 recrystallisations (methanol-ether).

Incorporation - less than 10^{-3}%

R,S- [1,17-$^3H_2$]-Erysodienone (LXXX) to E. berteroana.

The precursor (LXXX) (2.36mg., 2.80 ± 0.15 x 10^5 dps) was fed as its hydrochloride salt to one plant on 26/5/72 and harvested on 28/6/72 (weight of wet plant 3.51g). Inactive α-erythroidine hydrochloride (14.4mg) was added and the crude alkaloid (15.1mg) was separated. Active erythroidine hydrochloride was isolated.

Activity/dps mg^{-1}	Number of recrystallisations
14.4 ± 0.08	3
17.0 ± 0.09	4
13.8 ± 0.08	5

Incorporation 0.078 ± 0.006%

[17-^{3}H]- Erysodine (LXXVI) to E. berteroana.

[17-^{3}H]- Erysodine (LXXVI) (1.81mg., 1.40 ± 0.08 x10^5dps) was fed as its hydrochloride salt to one plant on 26/5/72 and harvested on 28/6/72 (plant weight 3.60g). Inactive α-erythroidine hydrochloride (15.4mg) was added and crude alkaloid (15.9mg) was separated. Active erythroidine as its hydrochloride salt was counted.

Activity/dps mg^{-1}	Number of recrystallisations
44.0 ± 3.0	3
44.0 ± 3.0	4
47.5 ± 3.0	5

Incorporation = 0.50 ± 0.04%

[14,17 - $^3H_2$]- Erysopine (LXVIII) to E. berteroana.

The precursor (LXVIII) (1.60mg., 1.81 ± 0.09 x 10^5 dps) was fed as its hydrochloride salt to one plant on 26/5/72 and harvested on 28/6/72 (6.00g). Inactive α-erythroidine hydrochloride (13.5mg) was added and crude alkaloid (14.8mg) was separated. Active erythroidine was isolated and counted as its hydrochloride salt.

Activity/dps mg^{-1}	Number of recrystallisations
28.0 ± 1.5	3
29.4 ± 1.5	4

Incorporation 0.21 ± 0.03%

Feedings to E. berteroana during 1971 Season using the Wick Method.

The method of feeding was essentially the same as described earlier except that as the plants were more sturdy and two positions on the plant were used to introduce the precursor. In the experiment when R,S-[2-^{14}C]-tyrosine was fed to two plants on 30/4/71, inactive β -erythroidine (LII) was added to the crude alkaloidal residue before separation of the erythroidines and crystallisation as the hydrochloride salt which was found to be inactive. In all cases the crude alkaloidal material isolated from the plant showed the presence of erythroidines according to t.l.c. The Table gives brief details of the feedings, and zero incorporation is less than the experimental limit of 5×10^{-3}%.

R,S-[2-^{14}C]-Tyrosine (L) to E. berteroana in 1971.

The precursor (0.3ml, $6.6 \pm 0.3 \times 10^{4}$ dps) was fed on 20/5/71 to one E. berteroana plant germinated in January 1969. The plant (144g) was harvested on 9/7/71 and inactive α - erythroidine (10.2mg) was added to the basic extract. Separation on t.l.c. (20% acetone-benzene) gave active erythroidine crystallised and counted as its hydrochloride salt.

Activity/dps mg^{-1}	Number of recrystallisations
0.84 ± 0.05	3
0.78 ± 0.05	4
0.89 ± 0.05	5

Incorporation 0.013 ± 0.001%

Table of feedings to E. berteroana during 1971 using the wick method

Precursor	Date fed	No. of plants used	Length of feeding	Age of plant	Activity fed/ 10^4dps	Incorporation /%
R,S-2-[^{14}C]-tyrosine(L)	30.4.71	2	4 days	1yr. 1mth.	6.6±0.3	0
"	30.4.71	2	4 days	2yr. 1mth.	6.6±0.3	0
"	20.5.71	1	7wk 1d	2yr. 4mth.	6.6±0.3	1.3±0.1 x 10^{-2}
[17-^{3}H]-Erysodine(LXXVI)	28.5.71	1	6 days	2yr. 2mth.	37.0±3.0	6.7±0.5 10^{-2}
R,S-[1,17-$^3H_2$]-erysodienone (LXXX)	11.6.71	1	7 days	3yr. 3mth.	16±1	0
[14,17-$^3H_2$]-erysopine (LXVIII)	21.6.71	1	11 dys	3 yr.	27.0±1.0	0.12±0.01
[17-^{3}H]-erysodine(LXXVI)	21.6.71	1	11days	3yr.	23.0±2.0	0
R,S-[2-^{14}C]-tyrosine(L)	15.7.71	2	5wk 4d	5mth.	6.6±0.3	0
R,S-[1,17-$^3H_2$]-erysodienone (LXXX)	15.7.71	2	7 days	5mth.	2.1±0.1	0
"	10.8.71	2	4wk 1d	5mth.	2.3±0.1	0
[14,17-$^3H_2$]-erysopine (LXVIII)	10.8.71	2	"	1yr. 5mth.	21.0±3.0	0
(±)-5,6,8,9-tetrahydro-2,12-[methyl-^{14}C]-dimethoxy-7H-[4,10-$^3H_2$]-dibenz[d,f]azonine-3,11-diol(XII)	10.8.71	1	5wk 3d	5mth.	7.1±0.4	0

[17-^{3}H]- Erysodine (LXXVI) to E. berteroana in 1971.

The precursor (2.0mg., 3.7 ± 0.3 x 10^5 dps) was fed as its hydrochloride salt to one plant germinated in March 1969. The whole plant (108g) was worked up on 3/6/71 and to the crude basic extract α -erythroidine (12.4mg) was added. Separation on t.l.c. gave α -/β- erythroidine which was crystallised and counted as its hydrochloride salt.

Activity/dps mg^{-1}	Number of recrystallisations
20.0 ± 1.0	4
20.0 ± 1.0	5

Incorporation 0.067 ± 0.005%

[14,17-$^3H_2$]-Erysopine (LXVIII) to E. berteroana in 1971.

The precursor (2.10mg., 2.7 ± 0.1 x 10^5 dps) was fed on 21/6/71 as its hydrochloride salt to one plant germinated in July 1968. Inactive α -erythroidine (11.6mg) was added to the crude basic extract and the α -/β- erythroidine mixture was separated on t.l.c. and crystallised as its hydrochloride salt.

Activity/dps mg^{-1}	Number of recrystallisations
27.0 ± 1.0	3
28.0 ± 1.0	4
28.0 ± 1.0	5

Incorporation 0.12 ± 0.01%

Cell Free Extract Type Feedings with E. berteroana.

Cell Free Extract Type Feeding using the Seeds[49].

Five E. berteroana seeds had a small part of the outer seed coating removed with a file and were then soaked for 2hr. in distilled water. The seeds were ground to a fine powder in a pestle and mortar which was surrounded by solid

carbon dioxide. Phosphate buffer (pH 7.0, 50ml) was added and the suspension was allowed to come to room temperature. R,S-[2-^{14}C]-Tyrosine (6.6 ± 0.3 x 10^4 dps) was added and the mixture shaken at 22°C for 24hr. The aqueous solution was centrifuged (1hr.), filtered through celite, made basic to pH 8.5 with solid sodium hydrogen carbonate and extracted with chloroform (6 x 20ml). Inactive β-erythroidine hydrochloride (7.9mg) was added and the chloroform was dried (Na_2SO_4) and removed under reduced pressure. The residue (20mg) was repeated on t.l.c. (ethyl acetate) to give inactive α-/β- erythroidine, crystallised and counted as the hydrochloride salt.

The residue from the filtration was extracted with 0.06M - hydrochloric acid (25ml) for 2 days at room temperature. The suspension was filtered through celite, basified with solid sodium hydrogen carbonate and extracted with chloroform (4 x 15ml). The combined chloroform extracts were dried over sodium sulphate and the solvent removed under reduced pressure. Inactive β-erythroidine was added and the mixture was separated on t.l.c. (ethyl acetate) to give α-/β erythroidine mixture. The erythroidine was crystallised and counted as its hydrochloride salt three times and found to be inactive.

Cell Free Extract Feeding to Germinating E. berteroana Seedlings[50]

Four E. berteroana seeds had a small piece of the seed coat removed, and were sterilised by dipping in 0.1% mercuric chloride solution and washing with sterilised water. The seeds were then placed on a sterile nutrient medium[82] containing 4% sucrose solution (25ml), stock salt solution (5ml)

and 0.1% ferrous sulphate solution (0.3ml) and allowed to germinate in the dark. After germination light was admitted. The nutrient medium and its container had been autoclaved at 10p.s.i. for 10 minutes. The stock salt solution had been prepared by dissolving anhydrous Ca $(NO_3)_2$ (10g), Na_2SO_4 (10g), KCl (4g), $NaH_2PO_4 . 2H_2O$ (0.95g), $MnSO_4\ 4H_2O$ (225mg), $ZnSO_4\ 4H_2O$ (134mg), H_3BO_3 (75mg) and KI (37mg) in distilled water (4l). After 12 days, the seedlings (c.a. 5cm. long) were crushed in a sterile pestle and mortar immersed in solid carbon dioxide. Using normal aseptic procedures, phosphate buffer solution (pH 7.4, 20ml) was added and the suspension centrifuged at 25° for 5 minutes. The supernatant was decanted, R,S-[2-^{14}C]-tyrosine (8.8 ± 0.4 x 10^4 dps) was added and the suspension was incubated for 15hr. at 29° with shaking. Inactive α-erythroidine hydrochloride (13.2mg) was added, the solution was acidified with 6M-hydrochloric acid (1ml), filtered through celite, made basic with solid sodium hydrogen carbonate and extracted with chloroform (6 x 50ml). The combined chloroform extracts were dried (Na_2SO_4), filtered and the solvent removed under reduced pressure. The residue (18.7mg) was separated on t.l.c. (20% acetone-benzene) to give the α-/β-erythroidine mixture which was crystallised as its hydrochloride salt, and counted (0.70mg). The erythroidine was inactive.

Other Methods of Feeding Attempted with E. berteroana and E. crista galli Seedlings.

Feeding via the medium using seedlings

The following experiments were carried out by adding the precursor to seedlings which were growing in a nutrient medium(34ml) consisting of 4% sucrose solution (29ml), stock

salt solution (3ml) and 0.1% ferrous sulphate solution (0.3ml). In all cases, t.l.c. indicated the presence of the respective alkaloid in the crude basic fraction. No activity was detected in the isolated α/β- erythroidine (LI & LII) (E. berteroana) or erythraline (XXVI) (E. crista galli).

Precursor	Activity 10^4dps	Age of Seedlings days	Number of Seedlings per experiment	Length of feeding days	Species	Comments
R,S-[2-^{14}C]-tyrosine	6.6±0.3	5	4	6	E. berteroana	Aseptic conditions
R,S-[1,17-$^{3}H_2$]-erysodienone	21±1	5	3	9	"	"
R,S-[2-^{14}C]-tyrosine	6.6±0.3	13	4	8	"	" + Roots split
"	6.6±0.3	10	4	25	"	
"	12.0±0.5	12	4	10	E. crista galli	Only water (c.a. 5ml) used as the medium

Feedings via the medium using a concentrated precursor solution.

General Method - A seed of both species (E. berteroana and E. crista galli) had a small part of the seed coat removed, was left in distilled water overnight and was allowed to germinate in the dark on a wet cotton wool surface (usually 2-3 days). Light was admitted and the seedling was left to grow at least 9 days before the precursor, R,S-[2-^{14}C]-tyrosine (8.8 ± 0.4 x 10^4 dps), was added. Beneath the surface of water, the root of the seedling was slit and transferred to a small polytop. The small polytop and the seedling were taken from the water and excess water inside the polytop was removed such that only the slit part of the root was still under water. The precursor solution was added and the seedling was left under an electric light for 9 hours per day. Distilled water was added as necessary to keep the liquid level above the split root.

Work up was carried out in the usual manner for E. berteroana and the isolated α-/β-erythroidine hydrochloride (LI and LII) was inactive. For E. crista galli, inactive erysodine (LXXV) (11.1mg) was added and erysodine was separated on t.l.c. (ethyl acetate), crystallised, counted as the free base and found to be inactive. The precursor solution which was left in the polytop was washed into a graduated flask (100ml) with absolute alcohol and two samples of this (1ml) had their solvent removed by a stream of nitrogen. The residue was counted.

	E. berteroana	*E. crista galli*
Precursor	R,S-[2-^{14}C]- tyrosine	R,S-[2-^{14}C]-tyrosine
Activity/dps x 10^4	8.8 ± 0.4	8.8 ± 0.4
Age of Seedling/ days	15	9
Length of Feeding/ days	6	6
Weight of seedling/g.	1.20	1.76
Weight of crude base separated/mg.	3.6 (0.3%)	13.0 (0.74%)
Activity remaining in the medium/ dps x 10^{-3}	4.7 ± 0.5 (5 ± 1%)	12 ± 1 (14±2%)
Incorporation	$<5 \times 10^{-3}$%	$<5 \times 10^{-3}$%

Experiment to determine the relative distribution of alkaloids against time in germinating E. crista galli seedlings.

Two E. crista galli seeds had a small part of the seed coating removed and were soaked in distilled water. They were germinated in the dark and then left on damp cotton wool in the laboratory. Two seeds were germinated at intervals such that all the seedlings could be harvested at the same time.

Work up - For each time interval, the two seedlings were crushed in a pestle and mortar and shaken for 3 days in 0.06M - hydrochloric acid (50ml). The suspension was filtered through celite, extracted once with light petroleum (20ml) and made basic with solid sodium hydrogen carbonate. The basic solution was extracted with chloroform (6 x 20ml). The combined chloroform extracts were dried (Na_2SO_4), filtered and the solvent was removed under reduced pressure to give crude basic material. Analar chloroform (1ml) was added to each extract and an equivalent amount of each solution was spotted side by side on a large t.l.c. plate (20cm x 20cm). Available samples of Erythrina alkaloids were also spotted on the appropriate plate. One such t.l.c. plate was run in a different solvent system (50% ethyl acetate - benzene, chloroform, chloroform-methanol 100:5, and chloroform-acetone-diethylamine 5:4:1-silica).

Age of Seedlings	Net Weight/g	Total weight of crude alkaloid extracted/mg	Percentage of alkaloid
0 (2 seeds)	0.96	20.3	2.1
1week 3days	2.24	12.4	0.55
2week 3days	2.54	5.1	0.20

Age of Seedlings	Net Weight/g	Total weight of crude alkaloid extracted/mg	Percentage of alkaloid
4 weeks	2.82	6.9	0.21
2 months	3.70	8.8	0.24

Observations - Erythraline (XXVI), erysodine (LXXV) and/or erysovine (LXXIV) (R_1=R_3=H, R_2=CH_3) were observed by co-t.l.c. and the intensities of the spots do not vary with time i.e. the amount of each alkaloid is constant despite the change the percentage of crude alkaloid.

There was an unidentified spot (R_f 0.53, 50% ethyl acetate-benzene) which, judging by U.V. intensity, decreased in amount with the age of the seedling.

There was an unidentified polar spot (R_f 0.37 chloroform-methanol 100-5) which was U.V. active and gave the usual crimson colour when sprayed with iodoplatinate. This spot was not present in the seed or the 2 month seedling extracts. The intensity of this spot was at a maximum after two weeks and three days.

Many unidentified polar spots (chloroform-acetone-diethylamine 5:4:1-silica) appeared after germination.

The seed extract was separated on t.l.c. (50% ethyl acetate-benzene) to give erythraline (XXVI), crystallised as its hydrobromide salt m.p. 247-249° (dec.) (lit.[3] m.p. 240-243°) and erysotrine[53] (LXXIV) (R_1=H, R_2=R_3=CH_3) (R_f 0.53) (1.1mg), which gave no colour with ferric chloride solution. It had a mass spectrum identical with the literature[68] of m/e M^+ 313, 298 and 282 (base peak); M^+ 313.1684 corresponds to 313.1678, $C_{19}H_{23}NO_3$; λ_{max} 227 and 280nm. The R_f of the

alkaloid was identical with that of the product of the reaction between erysodine (LXXV) and diazomethane.

The one week three day extract was separated on t.l.c. (analar chloroform) to give erysodine / erysovine which had peaks in the mass spectrum at m/e 299(M^+), 284 and 268 (base peak).

The whole experiment was repeated but only using one seed for each time interval and it was again observed that a spot (R_f c.a. 0.4 chloroform-methanol 100-5) was not present in the seeds or after one month thirteen days but was present in the extracts of seedlings of an intermediate age being a maximum after two weeks two days.

Repeat experiment (1 seedling).

Age of Seedlings	Net Weight/g	Total weight of crude alkaloid extracted/mg	Percentage of Alkaloid
0	0.34	9.8	3
9 days	0.83	6.5	0.78
2 weeks 2 days	1.5	6.5	0.43
1 month	2.0	4.4	0.22
1 month 13 days	0.90	3.8	0.42

Isolation of the alkaloid R_f 0.4 (chloroform-methanol 100:5) from E. crista galli seedlings.

E. crista galli seeds (20) had part of the seed coat removed and were soaked overnight in distilled water. They were germinated in the dark on damp cotton wool, and after the root had appeared they were left to grow in the light. The seedlings (24.12g) were harvested two weeks and three days after they were set, macerated in a pestle and mortar,

and shaken with 0.06M - hydrochloric acid (100ml) for 3 days under an atmosphere of nitrogen. The acidic suspension was filtered through celite, washed with light petroleum (20ml) and basified with solid sodium hydrogen carbonate. The basic solution was extracted with chloroform (6 x 20ml) and the combined extracts dried over sodium sulphate and filtered. The solvent was removed under reduced pressure to give crude alkaloid (49.9mg., 0.21%). The residue was separated on t.l.c. (chloroform-methanol 100:5) and the unknown alkaloid (5.8mg) (6% of crude base) was eluted from the alumina under an atmosphere of nitrogen. It was not possible to crystallise the free alkaloid (unstable), its hydrochloride salt or the picrate salt. It had significant peaks in the mass spectra at m/e 329, 313, 311, 310, 298, 297, 296, 295, 294, 282, 280 (base peak), 266 and 264. The hydrochloride (glassy solid) had $\lambda_{max.}$ [M^+ 329] 236 ($E^{\%}_{1cm}$ 500) and 287 ($E^{\%}_{1cm}$ 164) nm, unchanged by the addition of base and $\nu_{max.}$ (chloroform) 3400, 2420, 1620 and 1115cm^{-1}.

A sample of the compound was separated on t.l.c. and submitted for accurate mass measurement.

Peak/m/e	Measured	Calculated	Assigned
313	313.1678	313.1678	$C_{19}H_{23}NO_3$
313	313.1322	313.1313	$C_{18}H_{19}NO_4$
311	311.1516	311.1521	$C_{19}H_{21}NO_3$
298	298.1434	298.1443	$C_{18}H_{20}NO_3$
297	297.1365	297.1372	$C_{18}H_{19}NO_3$
282	282.1488	288.1494	$C_{18}H_{20}NO_2$

Peak/m/e	Measured	Calculated	Assigned
282	282.1134	282.1130	$C_{17}H_{16}NO_3$
280	280.1331	280.1337	$C_{18}H_{18}NO_2$
280	280.0995	280.0974	$C_{17}H_{14}NO_3$
266	266.1181	266.1181	$C_{17}H_{16}NO_2$
264	264.1018	264.1024	$C_{17}H_{14}NO_2$

The ratio $C_{19}H_{23}NO_3 / C_{18}H_{19}NO_4$ was approximately 5/1

and $C_{18}H_{20}NO_2 / C_{17}H_{16}NO_3$ was approximately 5/3

The isolated product gave no colour with ferric chloride and was still apparent as one spot after running three times on t.l.c. (chloroform-methanol 49:1).

Acetylation of the Unknown Compound - The glassy solid hydrochloride salt (c.a. 1mg) was left in redistilled pyridine (3 drops) and redistilled acetic anhydride under an atmosphere of nitrogen overnight at room temperature. The solvent was removed under reduced pressure and the gum was dried in vacuo. T.l.c. (chloroform-methanol 100:5) showed no spot corresponding to the unknown compound but there were 3 spots (chloroform) at R_f 0.8, 0.6 and 0.4. The mass spectrum of the crude product had significant peaks at m/e 371, 356, 355, 340, (base peak) 324, 312, 310, 296, 294, 280, 264.

Isolation of more product from E. crista galli [R_f 0.4 (chloroform-methanol 100:5)].

The procedure has been described above. Seedlings (wet

weight 39.57g) yielded crude alkaloid (120mg, 0.3%) which was seperated to give the unknown product (7.8mg, 6% of crude alkaloid). This was also still apparent as one spot on t.l.c. (chloroform-methanol, 49:1, 3 times). The free base had τ 3.30-3.70 (complex, aromatic - H), 3.87 (complex, olefinic proton), 4.00 (complex, olefinic proton), 4.14 (complex, olefinic proton), 4.29 (complex olefinic proton) 5.5-7.4 (broad complex, O-C-H and N-C-H), 6.21 (broad s, O-CH_3), 6.32 (broad s, O-CH_3), 6.72 (s, O-CH_3), 7.5-8.5 (complex, methylene protons). The integration gave aromatic:olefinic as 1.1 and the three O- methyl signals as equal. If there is one O-CH_3 (τ 6.72) then the olefinic/aromatic protons would integrate for four protons. It also had a specific rotation (589nm, 22°C) of + 105° dl. $g.^{-1}$ dm^{-1} (c = 0.544).

Attempted Oxidation with Active Manganese Dioxide.

The unknown (c.a. 1mg) was stirred with active manganese dioxide (5mg) in ethanol-free chloroform (1ml) under an atmosphere of nitrogen at room temperature. T.l.c. indicated that no new products had been formed and the U.V. spectrum of the product was identical to the starting material. The manganese dioxide was shown to be capable of oxidising benzyl alcohol to benzaldehyde in quantitative yield (t.l.c. and i.r.) in ethanol free chloroform after 7hrs. stirring at room temperature.

Erysonine (CXXXV)[70] - Erysodine (LXXV) (3mg) was heated at 100°C with 0.3M - hydrochloric acid for 2 hours. Separation of the crude product on t.l.c. (chloroform-methanol 57:4) gave erysonine (CXXXV) which had $\lambda_{max.}$ 225 (sh) and 284. There were significant peaks in the mass spectrum at m/e 285 (base

peak) and 268.

Erysodienols[81] (XC, all possibilities) - Erysodienone (XV) 6mg) was stirred in absolute ethanol with sodium borohydride (10mg) for 2 hours at room temperature. The solvent was removed under reduced pressure, water was added and the solution was extracted with chloroform. The combined chloroform extracts were dried (Na_2SO_4), filtered and evaporated to give a foam. This was a single spot on t.l.c. (chloroform-methanol 100:5) and the hydrochloride salt had $\lambda_{max.}$ 235 and 284, $\nu_{max.}$ (chloroform) 3540, 3380, 2500, 1650, 1600 cm^{-1}. The mass spectrum had significant peaks at m/e 315 (60%) and 298 (base peak). The free base had $\nu_{max.}$ (chloroform) 3540, 3280, 1650 cm^{-1}.

Experiment to determine the position of the label in α-/β-erythroidines (LI and LII) from E. berteroana feedings.

β - Erythroidinol[28] (LXXXV) - β - Erythroidine (LII) (21mg) in dry redistilled tetrahydrofuran (6ml) was refluxed for 3hr. with lithium aluminium hydride (6mg) under an atmosphere of nitrogen. Wet tetrahydrofuran was added, and the precipitate was filtered off and washed with hot tetrahydrofuran. The combined filtrate and washings was dried over sodium sulphate, filtered and the solvent removed under reduced pressure to give β-erythroidinol (LXXXV) (15.6mg., 74%) m.p. 168-170°, softening 163° (ether) (lit.[28] m.p. 168°, softening 163°). It showed $\nu_{max.}$ (chloroform), 3420, 1110 cm^{-1}, τ 3.60 (d. of d., 1-H, $J_{1,2}$ 10Hz, $J_{2,3}$ 2.5Hz, H-2), 4.16 (broad d, 1H, $J_{1,2}$ 10Hz, H-1), 4.23 (m, 1H, H-7), 5.5-6.8 (complex, 9-H, C-$\underline{H}_2$ and $>\overset{OCH_3}{C-\underline{H}}$), 6.64 (s, 3-H, O-$C\underline{H}_3$), 7.08 (m, 4-H, -C-$\underline{H}_2$), 8.0-8.5 (complex, 2H, H-4). The mass spectrum had significant peaks at m/e 277, 275, 262, 260, 245, 243, 228, 130 (base peak).

α -Erythroidinol[29] (LXXXIX) - α -Erythroidine (L1) (20.1mg.) was stirred in dry, redistilled ether (6ml) with lithium aluminium hydride (5mg) at room temperature for 2 hours. T.l.c. (chloroform-methanol 100:6) showed the presence of two new spots. Wet ether was added. The solid was filtered off and washed with hot ether. The combined filtrate and washings was dried (Na_2SO_4) and filtered to give a residue. The gum was separated on t.l.c. to give α -erythroidinol (LXXXIX) (10.1mg, 50%) as the more polar spot. It had m.p. 155-157° (ether) (lit.[29] m.p. 155-156° from benzene) and τ 3.72 (d. of d., 1-H, $J_{1,2}$ 11Hz, $J_{2,7}$ 2Hz, H-2), 4.22

(m, 1H, $J_{1,2}$ 11Hz, H-1), 4.34 (m, 1H, H-7), 4.62 (t, 1H, $J_{14,15}$ 7Hz, H-14), 5.86 (m, 2H, H-15), 5.94-6.36 (complex, 3H, H-17 and H-3), 6.47 (broad s, 2H, H-8), 6.70 (s, 3H, O-C$\underline{H}_3$), 6.96-7.50 (complex, 4H, H-10 and H-11), 8.00-8.70 (complex, 3H, H-4 and H-12). Irradation at τ5.86 reduced the triplet at τ4.62 to a singlet.

15-desoxy-17-oxo-$\Delta^{12,13}$-erythroidine (LXXXVII) - β-Erythroidinol (LXXXV) (44mg) was stirred in analar acetone (2ml) with a solution of 0.18M - chromic acid (7.1ml) (Jones reagent[83]) for 38 hours. The solution was made basic with solid sodium hydrogen carbonate and extracted with chloroform (4 x 5ml). The combined chloroform layers were dried over sodium sulphate, filtered and the solvent was removed under reduced pressure to give an oily residue. This was separated on t.l.c. (50% ethyl acetate-benzene and silica - 5% chloroform-methanol), to give the 15-desoxy-17-oxo-$\Delta^{12,13}$ erythroidine (LXXXVII) (11.1mg, 25%) as its hydrochloride monohydrate salt, colourless needles m.p. 190-193° (decomp.) (absolute ethanol). The hydrochloride monohydrate salt had $\nu_{max.}$ (nujol) 3450, 2500, 2450, 1708 cm^{-1}, $\lambda_{max.}$ 229nm., (12,000), $[\alpha]_D^{22}$ + 86° (methanol, c = 0.049) and had significant peaks in the mass spectrum at m/e 273 (M^+) 258, 243, 242 (base peak), 240 and 228. (Found C, 58.58; H, 6.68; N, 3.97 $C_{16}H_{22}NO_4Cl$ requires C, 58.63; H, 6.77; N, 4.27; O, 19.52; Cl, 10.82%). The one molecule of water of crystallisation was not removed by drying at 60°C in vacuo for 2.5 days. The free base had τ3.53 (broad d, 1H, $J_{1,2}$ 10Hz, H-2), 4.09 (m, 1H, $J_{1,2}$ 10Hz, H-1), 4.20 (m, 1H, H-7), 5.80-6.20 (complex, 3-H, H-15 and H-3), 6.40 (m, 2H, H-8),

6.60 (s, 3H, O-C$\underline{H}_3$), 6.60-7.20 (complex, 2H, -C$\underline{H}_2$), 7.40-8.50 (complex, 6H, -C-$\underline{H}_2$).

Isomerisation of α-erythroidine to β-erythroidine[27].

α-Erythroidine hydrochloride (LI) (25mg) was added to a 10% solution of analar sodium hydroxide in water (5ml) and refluxed for 3 hour under an atmosphere of nitrogen. Dilute hydrochloric acid was added to make the solution acidic and it was left at room temperature for two hours. Solid sodium hydrogen carbonate was added and the basic solution was extracted with chloroform (4x5ml). The combined extracts were dried (Na_2SO_4), filtered and the solvent removed under reduced pressure to give β-erythroidine (16.2mg, 65%) (LII) which had an n.m.r. spectrum completely superimposable with that of authentic material.

Experiment on Active Material from a Feeding (Scheme 1C, R=^{3}H)

[17-^{3}H]-Erythroidine hydrochloride (2.3mg, 45.1 dps mg^{-1}) from the feeding of [17-^{3}H]-erysodine (LXXVI) to E. berteroana for 4 weeks 5 days was further diluted by the addition of inactive α-erythroidine hydrochloride (18.0mg). The specific activity was now 5.1 dps mg^{-1}. This was now converted to β-erythroidine (13.3mg, 78%) (LXXXII) in the manner described above. Without further purification the β-erythroidine was reduced with lithium aluminium hydride (6mg) in dry tetrahydrofuran to β-erythroidinol (LXXXVI) (7.9mg, 60%) showing a single spot on t.l.c. (5% methanol-chloroform). Again without purification of the β-erythroidinol, it was oxidised by 4.5×10^{-4}M - chromic acid (0.36ml) in analar acetone (1ml) at room temperature after 3 days to a product which had six spots on t.l.c..

The major spot was separated on t.l.c. (50% ethyl acetate-benzene) to give 15-desoxy-17-oxo- $\Delta^{12,13}$-erythroidine (LXXXVII) (1.6mg, 20%) as its hydrochloride monohydrate salt. It was crystallised 3 times from absolute ethanol and counted (0.62mg). The specific activity was measured as 0.06 dps mg^{-1} (1%) which was at the limit of the sensitivity of the counter.

Therefore 99 $\pm$ 6 (est.) % of the activity was lost.

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