PERGAMON

Phytochemistry 52 (1999) 373–382

PHYTOCHEMISTRY

Biosynthesis of *Erythrina* alkaloids in *Erythrina crista-galli*

Ulrich H. Maier, Wolfgang Rödl, Brigitte Deus-Neumann, Meinhart H. Zenk*

Institut für Pharmazeutische Biologie, Ludwig-Maximilians-Universität München, Karlstrasse 29, 80333 Munich, Germany

Received 25 February 1999; accepted 31 March 1999

Abstract

A precursor application system was developed to allow the study of *Erythrina* alkaloid formation in *Erythrina crista-galli*. Fruit wall tissue of this species was recognized as the major site of alkaloid biosynthesis. The application of radioactively and ^{13}C-labelled potential precursors showed that the hitherto assumed precursor (*S*)-norprotosinomenine was not incorporated into the *Erythrina* alkaloids. In contrast, (*S*)-coclaurine as well as (*S*)-norreticuline were metabolized to erythraline and erythrinine, respectively, suggesting that a coclaurine-norreticuline pathway is operative in *Erythrina* alkaloid formation. Feeding of [1-^{13}C]-labelled (*S*)-norreticuline with subsequent NMR spectroscopy demonstrated that the resulting erythraline was exclusively labelled at position C-10. Therefore, the participation of a symmetrical intermediate of the diphenoquinone type in *Erythrina* alkaloid biosynthesis can be excluded. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Erythrina crista-galli*; Fabaceae; Biosynthesis; *Erythrina* alkaloids; Erythrinine; Erythraline; Application experiments

1. Introduction

Erythrina crista-galli L. (Fabaceae) is a popular ornamental plant in subtropical areas. Pharmacological investigations have demonstrated that *E. crista-galli* seed extracts possess sedative, hypertensive, laxative and diuretic activities (Greshoff, 1890). Moreover, the seed extracts of certain *Erythrina* species showed a curare-like effect on the central nervous system (Hider, Walkinshaw, & Saenger, 1986; Roth, Daunderer, & Kormann, 1994). The chemical constituents responsible for these pharmacological activities are the alkaloids of the *Erythrina* type, which possess an unique spirocyclic structure (Fig. 1).

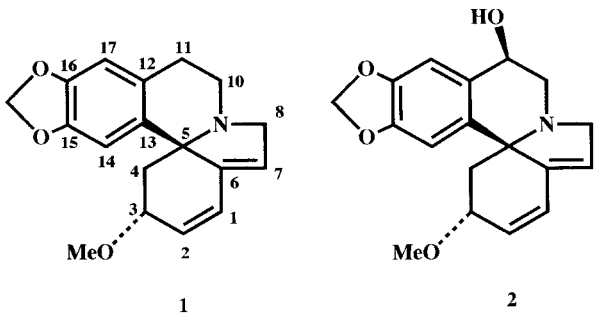
Barton and Cohen (1957) proposed the first biosynthetic pathway for the biosynthesis of this unusual class of alkaloids. Subsequently, they made the important experimental observation that *Erythrina* alkaloids are formed by skeletal rearrangement from benzylisoquinoline alkaloids (Barton, James, Kirby, Turner, & Widdowson, 1968). By feeding of potential precursors to young *E. crista-galli* plants it was observed that (*S*)-norprotosinomenine (**3**) (Fig. 2) was incorporated into the *Erythrina* alkaloid erythraline (**1**), but only to the relatively low extent of 0.25% (Barton, Boar, & Widdowson, 1970b; Barton et al., 1968; Barton, Potter, & Widdowson, 1974). Furthermore, application of norprotosinomenine (**3**) to the taxonomically remote species *Cocculus laurifolius* DC. (Menispermaceae) also resulted in the labelling of the *Erythrina* alkaloid coccuLidine (Bhakuni & Singh, 1978). Previously it had been demonstrated that (*S*)-norprotosinomenine (**3**) is a natural compound in *Erythrina variegata* L. (formerly *Erythrina lithosperma* BLUME) fruits (Ghosal, Majumdar, & Chakraborti, 1971). During their studies, Barton et al. (1974) applied structural isomers of norprotosinomenine (**3**) to whole *E. crista-galli* plantlets, but incorporation rates higher than 0.1% were never observed. Norprotosinomenine-based biomimetic syntheses of *Erythrina* alkaloids were either not successful (Barrett, Barton, Franckowiak, Papaioannou, & Widdowson, 1979) or not reproducible (Hewgill & Pass, 1985; Kupchan, Kim, & Lynn, 1976).

Numerous biosynthetic investigations have shown

* Corresponding author. Tel.: +49-89-5902-244; fax: +49-89-5902-611.

E-mail address: zenk@lrz.uni-muenchen.de (M.H. Zenk)

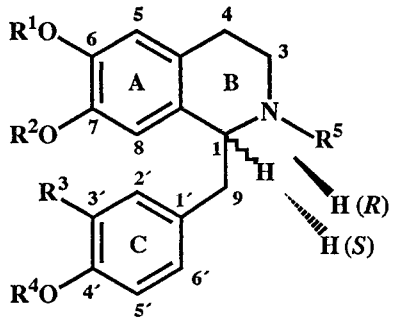
0031-9422/99/$ - see front matter
PII: S0031-9422(99)00230-7



1 2

Fig. 1. Structures of the *Erythrina* alkaloids erythraline (**1**) and erythrinine (**2**).

that a multitude of isoquinoline alkaloids belonging to the aporphine, benzophenanthridine, bisbenzylisoquinoline, morphinan, pavine and protoberberine type are derived from (*S*)-reticuline (**6**) and from its precursor (*S*)-norcoclaurine (**9**) (Zenk, 1995). These results have been verified on the enzymic (Gerardy & Zenk, 1992; Stadler & Zenk, 1993) as well as on the genetic level (Kutchan, 1998). The pathway of *Erythrina* alkaloid biosynthesis as proposed by Barton et al. (1968) with norprotosinomenine (**3**) as precursor would, therefore, be the only biosynthetic exception so far known. Since the enzymes utilizing (*S*)-reticuline (**6**) and (*S*)-norcoclaurine (**9**) are highly specific and do not accept norprotosinomenine (**3**) (Kutchan, 1998), the biosynthesis of *Erythrina* alkaloids would require a completely different set of enzymes, which on comparative biochemical grounds seemed unlikely to us.



Norprotosinomenine (**3**): $R^1 = H$; $R^2 = Me$; $R^3 = OH$; $R^4 = Me$; $R^5 = H$
Coclaurine (**4a**): $R^1 = Me$; $R^2 = H$; $R^3 = H$; $R^4 = H$; $R^5 = H$
4′-Glucosylcoclaurine (**4b**): $R^1 = Me$; $R^2 = H$; $R^3 = H$; $R^4 = Glc$; $R^5 = H$
Norreticuline (**5**): $R^1 = Me$; $R^2 = H$; $R^3 = OH$; $R^4 = Me$; $R^5 = H$
Reticuline (**6**): $R^1 = Me$; $R^2 = H$; $R^3 = OH$; $R^4 = Me$; $R^5 = Me$
Nororientaline (**7**): $R^1 = Me$; $R^2 = H$; $R^3 = OMe$; $R^4 = H$; $R^5 = H$
Norisoorientaline (**8**): $R^1 = H$; $R^2 = Me$; $R^3 = OMe$; $R^4 = H$; $R^5 = H$
Norcoclaurine (**9**): $R^1 = H$; $R^2 = H$; $R^3 = H$; $R^4 = H$; $R^5 = H$
Protosinomenine (**10**): $R^1 = H$; $R^2 = Me$; $R^3 = OH$; $R^4 = Me$; $R^5 = Me$
Orientaline (**11**): $R^1 = Me$; $R^2 = H$; $R^3 = OMe$; $R^4 = H$; $R^5 = Me$

Fig. 2. Structures of putative precursors of *Erythrina* alkaloid biosynthesis.

To verify whether this potential exception is realized and to reinvestigate the structure of the precursor for this unique group of spirocyclic *Erythrina* alkaloids, radioactively and ^{13}C-labelled potential precursors were applied to *E. crista-galli* plant material. Prior to these experiments, however, the physiological optimization of the application system was developed to achieve satisfactory rates of incorporation of potential precursors.

2. Results

2.1. Development of an application system

In previous studies Barton and coworkers (Barton et al., 1968, 1970b, 1974) found only small rates of incorporation when potential precursors were fed to four- to five-month-old *E. crista-galli* plants (Barton, Boar, & Widdowson, 1970a). Therefore we attempted to optimize the application system so as to obtain incorporation rates of potential precursors that allowed the use of ^{13}C-labelled compounds.

For this purpose, *E. crista-galli* plants were chosen to make results comparable with previously obtained data (Barton et al., 1968). Phytochemical investigations during the progress of our studies showed in accordance with earlier work (Barton et al., 1968) that *Erythrina* alkaloids are present in all parts of the plants. Erythraline (**1**) was determined as major alkaloid in leaves (0.032% dry wt), while in fruits 0.18% (dry wt) erythraline (**1**) and 0.015% (dry wt) erythrinine (**2**) were found. Seeds produced erysodine as main constituent with a 0.04% (dry wt) content. Therefore, every organ of the *Erythrina* plant may be a possible system for application experiments.

The erythraline (**1**) content of whole seedlings was analyzed and was shown to be almost constant (0.015% dry wt) in plants during a growth period of 2 to 4 weeks. Furthermore, cell suspension and callus cultures of *E. crista-galli* were established, but did not produce the alkaloids under investigation even after elicitation with methyl jasmonate.

The radioactively labelled potential precursors listed in Table 1 were applied to whole seedlings and to root, stem, leaf and flower tissue using the cotton wick method as previously used (Barton et al., 1968). As well the potential precursors were allowed to be taken up by the transpiration stream. The application of 0.5 μCi of each of the ^{3}H-labelled precursors in a 0.01 mM solution (0.5 ml) for 7 days, however, was not successful in labelling of erythraline (**1**) (incorporation $< 0.0001\%$).

Analysis of the tissue that had taken up the labelled potential precursor showed that up to 90% of the radioactivity applied was found in the region of appli-

Table 1
Feeding of radioactively labelled precursors to *Erythrina crista-galli* seedlings. The labelled precursors (0.5 μCi) were used at 0.01 mM concentration (0.5 ml) and were applied to the seedlings using the cotton wick method. Incubation was for 7 days at 20°C. *Radioactivity remaining in the incubation vessel after 7 days

Precursor	Specific activity (Ci mol^{-1})	Radioactivity in crude extract (%)	Radioactive residue* (%)
(*R*)-[7-OC^{3}H$_3$]Norprotosinomenine	100	68	10
(*S*)-[7-OC^{3}H$_3$]Norprotosinomenine	100	77	13
[6-OC ^{3}H$_3$]Nororientaline	100	74	9
(*R*)-[6-OC^{3}H$_3$]Norreticuline	100	72	10
(*S*)-[6-OC^{3}H$_3$]Norreticuline	100	76	9
[7-OC^{3}H$_3$]Norisoorientaline	100	74	12
(*R*)-[6-OC^{3}H$_3$]Coclaurine	100	76	14
(*S*)-[6-OC^{3}H$_3$]Coclaurine	100	71	10

cation. Transport experiments using ^{14}C-labelled (*S*)-coclaurine (**4a**) also demonstrated that this substance only entered ca. 0.5 mm into the bundle sheaths of the stem used. The precursors were obviously not transported to any extent into areas outside the application fluid.

As a consequence of this result, leaf, stem and fruit wall tissue was cut into slices (3–5 mm thick), which were allowed to float separately on the aqueous tracer solutions (0.5 μCi) given in Table 1 under constant shaking for 24 h. No potential precursors were incorporated into erythraline (**1**) when applied to leaf tissue of *E. crista-galli*. Surprisingly, fruit tissue showed considerable incorporation of (*S*)-norreticuline (**5**) and (*S*)-coclaurine (**4a**) into both erythraline (**1**) (7.9 and 11.7%, respectively) and erythrinine (**2**) (12.1 and 13.2%, respectively) (Table 2). Their corresponding (*R*)-configurated congeners were not incorporated into the alkaloids under investigation. Very surprisingly, the hitherto postulated intermediate of *Erythrina* alkaloid biosynthesis, (*S*)-norprotosinomenine (**3**), was not converted into any of the alkaloids investigated (**1** and **2**).

One-dimensional TLC analysis of the fruit wall extracts from application experiments using (*S*)-[7-OC^{3}H$_3$]norprotosinomenine (**3**) and (*S*)-[6-OC^{3}H$_3$]coclaurine (**4a**) as shown in Fig. 3 confirmed these findings. While no further labelled substance was detected in the extract after feeding of labelled norprotosinomenine (**3**), (*S*)-[6-OC^{3}H$_3$]coclaurine (**4a**) was converted to labelled erythraline (**1**) and erythrinine (**2**) as well as into some other products at a relatively high incorporation rate. In contrast, absolutely no incorporation into the *Erythrina* alkaloids was observed when the labelled substances were fed to leaf tissue of *E. crista-galli*.

Obviously, the cells of the fruit wall are capable of synthesizing *Erythrina* alkaloids. Therefore, sliced fruit wall tissue was chosen for further application experiments.

Since the stage of ripening may influence the extent to which alkaloids are synthesized, fruit wall tissue of different stages of maturity was now used for incubation with (*S*)-[6-OC^{3}H$_3$]coclaurine (**4a**). For this purpose, three fruit stages with increasing size and different alkaloid content were chosen. While the alkaloid content in capsule stage I (size: 3–6 cm in length) was comparatively low (0.01–0.03% dry wt), the con-

Table 2
Feeding of radioactively labelled precursors to *Erythrina crista-galli* fruit wall tissue. The labelled precursors (0.5 μCi) were used at 0.01 mM concentration (0.5 ml). The sliced fruit wall tissue (16–23 mg fr. wt; 0.47–0.56 mg dry wt) was allowed to float separately on the tracer solutions under shaking at 22°C for 24 h. Specific activity of the precursors: see Table 1

Precursor	Radioactivity in crude extract (%)	Incorporation into erythraline (%)	Incorporation into erythrinine (%)
(*R*)-[7-OC^{3}H$_3$]Norprotosinomenine	76	0	0
(*S*)-[7-OC ^{3}H$_3$]Norprotosinomenine	71	0	0
[6-OC^{3}H$_3$]Nororientaline	86	0	0
(*R*)-[6-OC^{3}H$_3$]Norreticuline	79	0	0
(*S*)-[6-OC^{3}H$_3$]Norreticuline	76	7.9	12.1
[7-OC^{3}H$_3$]Norisoorientaline	78	0	0
(*R*)-[6-OC^{3}H$_3$]Coclaurine	88	0	0
(*S*)-[6-OC^{3}H$_3$]Coclaurine	84	11.7	13.2
(*R*,*S*)-[6-OC^{3}H$_3$]Reticuline	74	0	0
(*R*,*S*)-[6-OC^{3}H$_3$]Orientaline	79	0	0
(*R*,*S*)-[7-OC^{3}H$_3$]Protosinomenine	77	0	0

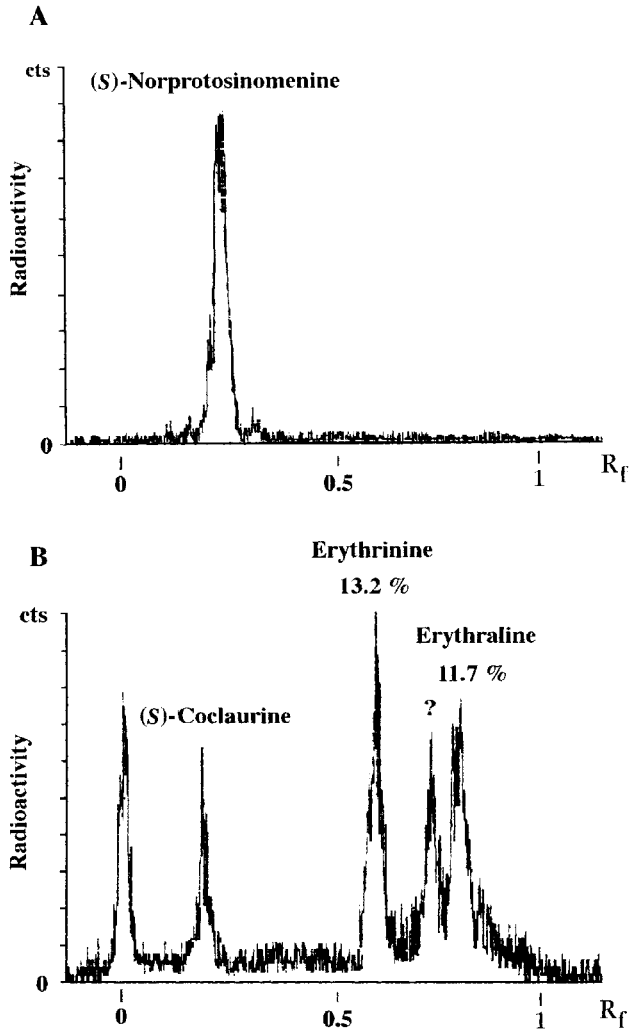


Fig. 3. Radioscan of *Erythrina* fruit wall extracts after TLC separation from application experiments with (A) (*S*)-[7-OC^3H_3]norprotosinomenine (**3**) and (B) (*S*)-[6-OC^3H_3]coclaurine (**4a**).

tent of erythraline (**1**) increased to 0.2% in stage II (6–11 cm) followed by a drastic decrease to less than 0.01% in stage III (11–16 cm) prior to seed ripening.

Sliced fruit walls of these various stages were incubated separately with ^{3}H-labelled (*S*)-coclaurine (**4a**). At the end of incubation it was observed that the uptake of radioactivity was correlated with the alkaloid content of the stage of maturity. The analysis of the labelling of erythraline (**1**) and erythrinine (**2**) confirmed that the highest amount of radioactivity was found in the alkaloids of fruit walls belonging to stage II, where 8.9 and 8.5% incorporation was found. Fruit wall slices of stages I and III had incorporated only up to 2.5% of the applied radioactivity. According to this result, wall tissue from capsules of stage II (size 6–11 cm in length) was chosen for further application experiments.

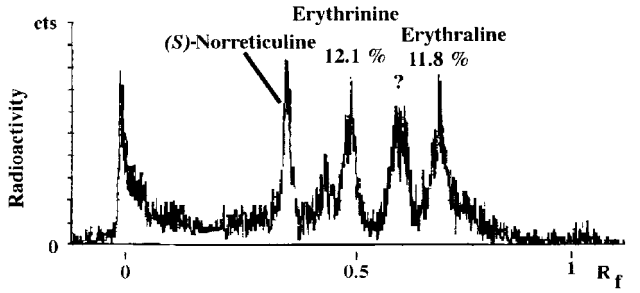


Fig. 4. Radioscan of *Erythrina* fruit wall extracts after TLC separation from application experiments with (*S*)-[6-OC^3H_3]norreticuline (**5**).

For further optimization, the dependence of (*S*)-coclaurine (**4a**) incorporation on substrate concentration was measured. Feeding of (*S*)-[6-OC^3H_3]coclaurine (**4a**) in concentrations higher than 0.02 mM reduced the incorporation rate into erythrinine (**2**) from more than 13% to 8–12%. The incorporation into erythraline (**1**) was almost constant at 12% for substrate concentrations of 0.01–0.05 mM. Addition of 0.1 mM of labelled substrate led to incorporation rates in the range of 4–6%. Further addition of (*S*)-coclaurine (**4a**) up to 1 mM reduced the incorporation of radioactivity to a minimum ($<0.1\%$). The results indicate that there is substrate inhibition of the enzymes responsible for *Erythrina* alkaloid biosynthesis.

According to the results described above the following conditions were chosen for an optimal application system: 3–4 slices of 3–5 mm thickness (fr. wt: 15–25 mg; dry wt: 0.16–0.27 mg per slice) from fruit walls of stage II (fruit size: 6–11 cm) were floated on an aqueous 0.05 mM substrate solution with rotary shaking for 24–36 h. Using this optimized application method, the incorporation of ^{3}H-labelled isoquinoline alkaloid precursors into erythraline (**1**) could be increased more than 100-fold in relation to the results obtained previously (Barton et al., 1968, 1970a,b, 1974).

2.2. Application of ^{3}H-labelled potential precursors

With the new application system in hand, different ^{3}H-labelled potential precursors of *Erythrina* alkaloid biosynthesis were administered to *E. crista-galli* fruit wall slices. In addition to all four norprotosinomenine isomers (**3**, **5**, **7** and **8**), (*R*)- and (*S*)-coclaurine (**4a**) as well as (*S*)-4′-β-glucosylcoclaurine (**4b**), which is less sensitive for oxidation and which should be transported into the cells more easily, were chosen for these experiments. Moreover, the key intermediate reticuline (**6**) and its isomers **10** and **11** were applied. After incubating for 24 h the *Erythrina* tissue was worked up

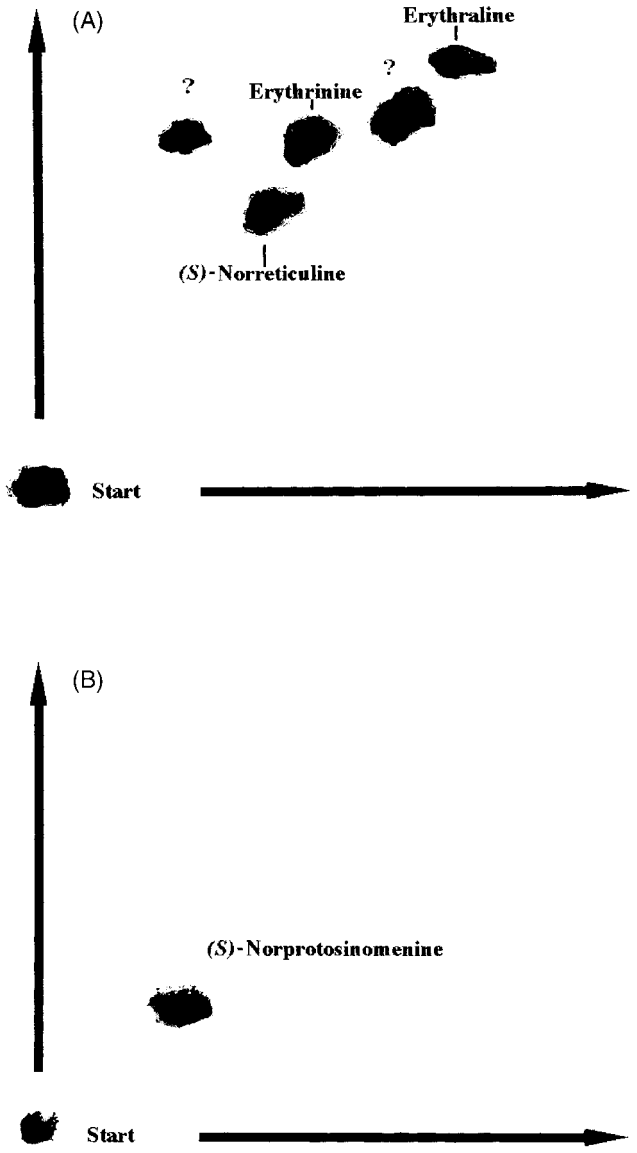


Fig. 5. Autoradiography of *Erythrina* fruit wall extracts after two-dimensional TLC separation from application experiments with (A) (*S*)-[6-OC^{3}H$_{3}$]norreticuline (**5**) and (B) (*S*)-[7-OC^{3}H$_{3}$]norprotosinomenine (**3**).

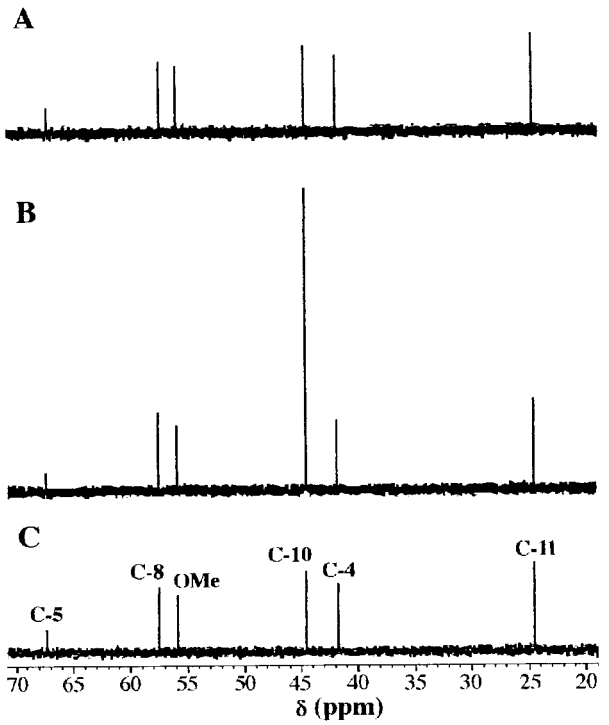


Fig. 6. Partial proton-decoupled ^{13}C NMR spectrum of erythraline (**1**) isolated after application of (A) (*S*)-[1-^{13}C]norprotosinomenine (**3**) and (B) (*S*)-[1-^{13}C]norreticuline (**5**); (C) authentic reference sample.

and the extracts analyzed as described in the Experimental.

The applied precursors were taken up very efficiently and showed rates of uptake of 70–80% while 7–14% of the radioactivity remained in the incubation vessels. Separation of the fruit wall extracts by TLC after precursor application again demonstrated that the hitherto postulated precursor of *Erythrina* alkaloids, (*S*)-norprotosinomenine (**3**) (Barton et al., 1968, 1970b, 1974), was not incorporated into erythraline (**1**) and erythrinine (**2**). Nororientaline (**7**), which has been isolated from various *Erythrina* species (Barton, Gunatilaka, Letcher, Lobo, & Widdowson, 1973; Ito, Haruna, Jinno, & Furukawa, 1976), was also not metabolized (Table 2). A significant labelling of *Erythrina* alkaloids was found only when (*S*)-norreticuline (**5**) or (*S*)-coclaurine (**4a**) were fed to the fruit wall tissue. Application of (*S*)-norreticuline (**5**) gave incorporation rates of 11.8% (erythraline) and 12.1% (erythrinine) (Fig. 4), while feeding of (*S*)-coclaurine (**4a**) resulted in 11.3 and 11.8% labelling of erythraline (**1**) and erythrinine (**2**), respectively. In contrast, the (*R*)-enantiomers of these precursors were not converted into the alkaloids under investigation. (*S*)-4′-β-Glucosylcoclaurine (**4a**) gave incorporation rates of 7.9% (erythraline) and 8.6% (erythrinine), which were lower than those of the unsubstituted precursor. Glucosylation, therefore, does not affect transport and incorporation. Since ^{3}H-labelled (*R*,*S*)-reticuline (**6**), (*R*,*S*)-protosinomenine (**10**) and (*R*,*S*)-orientaline (**11**) were not incorporated into the *Erythrina* alkaloids, *N*-demethylation of these compounds did not occur. The other possible precursors tested, (*R*)-norprotosinomenine (**3**) and (*R*,*S*)-norisoorientaline (**8**), were not metabolized into erythraline (**1**) and erythrinine (**2**), respectively.

To gain additional insight into the metabolism of the radioactively labelled precursors into the *Erythrina* alkaloids, fruit wall preparations were fed ^{3}H-labelled (*S*)-norreticuline (**5**) and (*S*)-norprotosinomenine (**3**). The resulting extracts were separated by 2D-TLC and subsequently analyzed by autoradiography. As

depicted in Fig. 5, the *Erythrina* alkaloids found in the extracts were only labelled when (*S*)-[6-OC^3H_3]norreticuline (**5**) had been fed to the fruit wall tissue (Fig. 5A). This labelled precursor was not only incorporated into erythraline (**1**) and erythrinine (**2**) but also into two additional compounds which may be intermediates of *Erythrina* alkaloid biosynthesis. In contrast, no labelled substances other than (*S*)-norprotosinomenine (**3**) were found in the extracts when this alkaloid was applied to the *Erythrina* tissue (Fig. 5B).

2.3. Feeding of ^{13}C-labelled compounds

The non-random participation of the above assumed precursors, (*S*)-coclaurine and (*S*)-norreticuline, in the biosynthesis of *Erythrina* alkaloids had to be experimentally proven. Furthermore, the participation of a symmetrical intermediate in erythraline biosynthesis as published previously (Barton et al., 1970a, 1974) required further investigation. For this purpose ^{13}C-labelled compounds were synthesized by standard methods (Barton et al., 1970a; Kratzl & Billek, 1951; Stadler & Zenk, 1990) and were fed at a 0.1 mM concentration to *E. crista-galli* fruit wall tissue on a large scale. After 24 h of incubation, the resulting erythraline (**1**) was isolated and analyzed by NMR spectroscopy.

The spectroscopic investigation established that only labelled (*S*)-norreticuline (**5**), (*S*)-coclaurine (**4a**) and (*S*)-4′-β-glucosylcoclaurine (**4b**) had been incorporated into **1** in a rate of 9.3, 5.5 and 6.3%, respectively. Feeding of (*S*)-[1-^{13}C]norprotosinomenine (**3**) yielded unlabelled erythraline (**1**) (Fig. 6A) (Maier & Zenk, 1997).

The ^{13}C NMR spectrum of erythraline (**1**) after incorporation of (*S*)-[1-^{13}C]norreticuline (**5**) as shown in Fig. 6(B) confirmed the intact incorporation of the applied precursor into the target alkaloid. The labelled erythraline (**1**) was characterized by an enhanced signal of C-10 due to the incorporation of the precursor ^{13}C-labelled at position 1. In addition, feeding of (*S*)-[1-^{13}C]coclaurine (**4a**) resulted in the isolation of **1** that was exclusively labelled at position C-10. If a symmetrical intermediate would occur in the biosynthesis of *Erythrina* alkaloids as had been postulated (Barton et al., 1970a, 1974), feeding of ^{13}C-labelled (*S*)-coclaurine (**4a**) and (*S*)-norreticuline (**5**) would have resulted in a ^{13}C-enrichment both at position C-10 and C-8 of erythraline (**1**). Since no ^{13}C-enrichment occurred at C-8, the participation of a symmetrical intermediate of the diphenoquinone type in *Erythrina* alkaloid biosynthesis can be excluded.

3. Discussion

The first feeding experiments of radioactive potential precursors (Barton et al., 1968) showed that (*S*)-norprotosinomenine (**3**) is the precursor to the spirocyclic alkaloid erythraline (**1**) in *Erythrina crista-galli* plants. A biomimetic synthesis conducted by Franck and Teetz (1971) demonstrated, however, that (*S*)-norreticuline (**5**), a positional isomer of norprotosinomenine (**3**), would on biomimetic grounds more likely be a precursor in *Erythrina* alkaloid biosynthesis. A biosynthetic sequence derived from (*S*)-norreticuline (**5**) would imply a morphinandienone intermediate rather than a neoproaporphine as proposed by Barton et al. (1968). By acid catalysis, this morphinandienone could be converted to a dibenz[*d*,*f*]azonine and finally oxidized yielding a symmetrical intermediate as postulated before (Barton et al., 1968; Franck & Teetz, 1971).

Extensive feeding experiments with all four possible isomers of (*S*)-norprotosinomenine (**3**) to *E. crista-galli* plantlets demonstrated that (*S*)-norprotosinomenine (**3**) was incorporated into erythraline (**1**) to a low extent (0.25%) (Barton et al., 1974). Obviously, the application system used by the Imperial College group, who applied putative precursors to five-month-old differentiated plants of *E. crista-galli* was not suitable for biosynthetic investigations and the incorporation rates were consequently marginal.

In the course of our studies, an optimized application system was developed that used *E. crista-galli* fruit wall tissue of a certain and defined stage of maturity. Feeding of radioactively labelled potential precursors under optimized conditions and isolation of erythraline succeeded in satisfactory incorporation rates 8–12% for (*S*)-[6-OC^3H_3]norreticuline (**5**) and 12% for (*S*)-[6-OC^3H_3]coclaurine (**4a**). Compared with earlier results (Barton et al., 1974), the incorporation rates achieved here could be improved by several orders of magnitude. Glucosylation of the precursor did neither increase the rate of transport nor the rate of incorporation. The hitherto assumed key intermediate in *Erythrina* alkaloid biosynthesis, (*S*)-norprotosinomenine (**3**), was clearly not incorporated at all into erythraline (**1**) nor were any of the additional isomers listed in Table 2. The results obtained from feeding experiments with radioactively labelled compounds clearly demonstrated that (*S*)-norreticuline (**5**) and not (*S*)-norprotosinomenine (**3**) is the committed precursor for the biosynthesis of *Erythrina* alkaloids.

The results obtained from precursor feeding experiments using ^{13}C-labelled compounds supported the conclusions which were drawn from the application of radioactively labelled substrates. NMR spectroscopy of the ^{13}C-labelled erythraline (**1**) obtained from [1-^{13}C]norreticuline clearly demonstrated a non-ran-

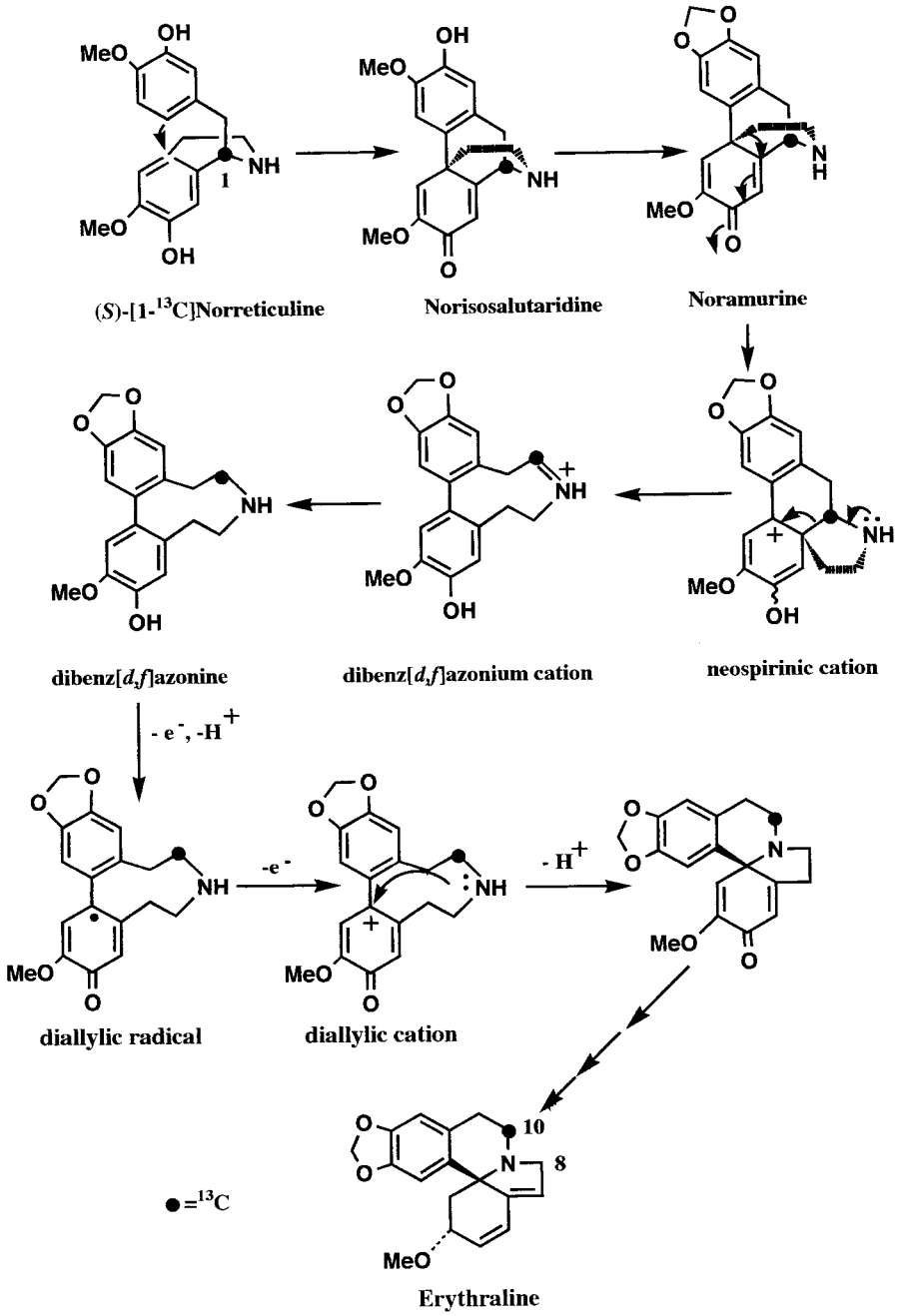


Fig. 7. Proposed new reaction sequence in the biosynthesis of *Erythrina* alkaloids in *Erythrina crista-galli*.

dom incorporation shown by an enrichment exclusively in position C-10 of the target molecule. Surprisingly, no label was found in position C-8, which would have been expected if an symmetrical intermediate (Barton et al., 1970a, 1974) would occur in the biosynthetic pathway. One can therefore exclude a symmetrical intermediate of the diphenoquinone type in the biosynthesis of *Erythrina* alkaloids.

The interpretation of the above results requires a new biosynthetic pathway and mechanism for the *Erythrina* alkaloid biosynthesis. Following an earlier proposal by Franck and Teetz (1971) (*S*)-norreticuline could be transformed by *para-para* phenol coupling to a morphinandienone, such as norisosalutaridine, which could, after formation of a methylenedioxy group at the aromatic ring, rearrange to an asymmetric dibenz[*d,f*]azonine. The dibenz[*d,f*]azonine could be oxidized in a two-electron process at the free phenolic ring to an asymmetric benzoquinone that possesses the chemical properties and reactivity of an allylic cation. It is assumed that it could react easily with the free electron pair of the nitrogen atom leading to the ery-

thrinane ring system (Fig. 7). The subsequent steps are similar to the mechanism already proposed by Barton et al. (1968).

4. Experimental

4.1. General

All NMR spectra were obtained on a Bruker AM 360 spectrometer. CD_3OD was used as an int. standard. ^1H NMR and ^{13}C NMR spectra were measured at 360 and 90.6 MHz, respectively. Mass spectra were recorded on a Finnigan-MAT SSQ 700 mass spectrometer. Distribution of radioactivity on TLC plates was monitored with a Berthold (Tracemaster 20) linear analyzer. The distribution of radiolabelled products on 2D TLC plates was determined with XAR-5 18 × 24 films (Kodak). The plates were sprayed with ^3H-Enhancer prior to exposing for 8 weeks.

4.2. Plant material

Seeds of *Erythrina crista-galli* were purchased from Bornträger and Schlemmer and were germinated under greenhouse conditions. A limited number of fruits were provided by the Botanical Garden, Munich. Further feeding experiments were conducted with an *E. crista-galli* plant at the Botanical Garden, Pisa, Italy.

Plant cell cultures of *E. crista-galli* were provided by the cell culture laboratory of this department.

4.3. Chemicals

All solvents and reagents were of the highest purity commercially available. Unlabelled reference substances were from the collection of this department. Catechol-*O*-methyltransferase (COMT) isolated from pork liver as well as (*S*)-[C^3H_3]adenosylmethionine (SAM) were kindly provided by Dr. M. Rüffer (Munich).

4.4. Synthesis of radioactively labelled precursors

Benzyltetrahydroisoquinoline alkaloids were enzymically methylated with [C^3H_3]SAM as a substrate. For this purpose, 50 nmol of the respective unlabelled precursor was incubated with 0.25 mmol sodium ascorbate, 20 μCi [C^3H_3]SAM (13.3 and 13.0 mCi μmol^{-1}, 1.5 and 1.55 nmol, respectively), 50 μg COMT and 0.25 mmol Tricin buffer, pH 7.5, in a total volume of 1 ml for 3–4 h at 37°C. The reaction was terminated by the addition of sodium carbonate buffer, pH 9.0, and the mixt. extracted with EtOAc. The products were purified by TLC (Polygram Sil G/UV_{254}, Macherey and Nagel, $CHCl_3$–MeOH–NH_3, 90:9:1; $CHCl_3$–MeOH–NH_3, 68:18:0.6).

4.5. Synthesis of ^{13}C-labelled precursors

^{13}C-labelled compounds were synthesized according to Kratzl and Billek (1951), Barton et al. (1970a) as well as Stadler and Zenk (1990) but using [^{13}C]KCN instead of KCN.

4.5.1. (*S*)-[1-^{13}C]Norreticuline HCl (**5**)

^1H NMR (CD_3OD): δ; 2.7–3.43 (6H, *m*, H-3, H-4, H-9), 3.73 (3H, *s*, 4′-OMe), 3.76 (3H, *s*, 6-OMe), 4.48 (1H, *d*, $J13_{C\text{-}H}$ = 111 Hz, H-1), 6.54 (1H, *d*, $J13_{C\text{-}H}$ = 4.3 Hz, H-8), 6.61 (1H, *d*, *J* = 8.1 Hz, H-5′), 6.64 (1H, *s*, H-5), 6.66 (1H, *s*, H-2′), 6.80 (1H, *d*, *J* = 8.1 Hz, H-6′). ^{13}C NMR (CD_3OD): δ; 29.2 (C-4), 41.4 (C-3), 43.4 (C-9, $^1J_{C\text{-}9/C\text{-}1}$ = 34.5 Hz), 54.9 (4′-OMe), 55.8 (6-OMe), 57.3 (C-1), 111.5 (C-2′), 113.8 (C-8), 114.2 (C-5′), 117.7 (C-5), 121.3 (C-6′), 123.3 (C-4a), 130.0 (C-8a, $^1J_{C\text{-}8a/C\text{-}1}$ = 42.1 Hz), 130.3 (C-1′), 145.0 (C-4′), 145.3 (C-6), 146.3 (C-3′), 146.7 (C-7). Yield: 32.3 mg.

4.5.2. (*S*)-[1-^{13}C]Norprotosinomenine HCl (**3**)

^1H NMR (CD_3OD): δ; 2.86–3.36 (6H, *m*, H-3, H-4, H-9), 3.67 (3H, *s*, 7-OMe), 3.76 (3H, *s*, 4′-OMe), 4.49 (1H, *d*, $J13_{C\text{-}H}$ = 120 Hz, H-1), 6.55 (1H, *s*, H-5), 6.63 (1H, *d*, $J13_{C\text{-}H}$ = 4.0 Hz, H-8), 6.74 (1H, *s*, H-2′), 6.75 (*d*, *J* = 8.0 Hz, H-6′), 6.82 (1H, *d*, *J* = 8.0 Hz, H-5′). ^{13}C NMR (CD_3OD): δ; 25.7 (C-4), 40.4 (C-3), 40.7 (C-9, $^1J_{C\text{-}9/C\text{-}1}$ = 35.1 Hz), 56.3 (OMe), 56.5 (OMe), 57.7 (C-1), 111.1 (C-2′), 113.2 (C-8), 116.1 (C-5′), 117.6 (C-5), 121.9 (C-6′), 123.4 (C-4a), 124.9 (C-1′), 129.4 (C-8a, $^1J_{C\text{-}8a/C\text{-}1}$ = 44.6 Hz), 147.7 (C-6), 148.1 (C-4′), 148.2 (C-3′), 148.7 (C-7). Yield: 26.6 mg.

4.5.3. (*S*)-[1-^{13}C]Nororientaline HCl (**7**)

^1H NMR (CD_3OD): δ; 2.73–3.41 (6H, *m*, H-3, H-4, H-9), 3.87 (6H, *s*, 6-OMe, 3′-OMe), 4.39 (1H, *d*, $J13_{C\text{-}H}$ = 119 Hz, H-1), 6.58 (1H, *d*, $J13_{C\text{-}H}$ = 4.3 Hz, H-8), 6.72 (1H, *s*, H-2′), 6.74 (1H, *d*, *J* = 7.5 Hz, H-5′), 6.84 (1H, *s*, H-5), 6.86 (*d*, *J* = 7.5 Hz, H-6′). ^{13}C NMR (CD_3OD): δ; 29.6 (C-4), 41.2 (C-3), 42.0 (C-9, $^1J_{C\text{-}9/C\text{-}1}$ = 36.3 Hz), 54.9 (OMe), 55.9 (OMe), 56.8 (C-1), 111.0 (C-5), 111.9 (C-2′), 114.4 (C-5′), 115.6 (C-8), 123.5 (C-6′), 126.3 (C-4a), 131.7 (C-8a, $^1J_{C\text{-}8a/C\text{-}1}$ = 44.8 Hz), 132.1 (C-1′), 144.2 (C-7), 145.0 (C-4′), 146.5 (C-6), 146.7 (C-3′). Yield: 33.4 mg.

4.5.4. (*S*)-[1-^{13}C]Coclaurine HCl (**4a**)

^1H NMR (CD_3OD): δ; 2.70–3.36 (6H, *m*, H-3, H-4, H-9), 3.83 (3H, *s*, 6-OMe), 4.39 (1H, *d*, $J13_{C\text{-}H}$ = 102 Hz, H-1), 6.59 (1H, *d*, $J13_{C\text{-}H}$ = 4.1 Hz, H-8), 6.63 (2H, *d*, *J* = 8.4 Hz, H-3′, H-5′), 6.79 (1H, *s*, H-5), 7.02 (2H, *d*, *J* = 8.4 Hz, H-2′, H-6′). ^{13}C NMR (CD_3OD): δ; 29.1 (C-4), 42.0 (C-3), 43.4 (C-9, $^1J_{C\text{-}9/C\text{-}1}$ = 34.5 Hz),

55.9 (6-OMe), 57.8 (C-1), 112.9 (C-8), 114.2 (C-5), 116.3 (C-3′/C-5′), 124.8 (C-4a), 129.7 (C-8a, $^{1}J_{C\text{-}8a/C\text{-}1}$ = 46.3 Hz), 130.5 (C-1′), 131.6 (C-2′/C-6′), 145.8 (C-7), 146.9 (C-6), 154.4 (C-4′). Yield: 48.6 mg.

(*S*)-[1-^{13}C]4′-β-Glucosylcoclaurine (**4b**) was synthesized analogously to coclaurine but with a methoxyethoxymethyl (MEM) protected derivative according to Corey, Gras and Ulrich (1976). The intermediate, (*S*)-[1-^{13}C]2,7-*N,O*-dibenzyl-4′-*O*-MEM-coclaurine (340 mg, 0.61 mmol), was dissolved in 10 ml CH_2Cl_2, 2 ml trifluoroacetic acid was added and the soln stirred for 5 h at room temperature. Subsequently, the reaction mixt. was washed ×3 with a total of 15 ml diluted NH_3 and ×3 with a total of 60 ml H_2O, dried over Na_2SO_4 and the solvent evapd yielding 240 mg (0.52 mmol) (*S*)-[1-^{13}C]2,7-*N,O*-dibenzylcoclaurine.

The above product was dissolved in 10 ml Me_2CO, and 75 mg (0.27 mmol) dried Ag_2CO_3 as well as 80 mg 2,3,4,6-tetra-*O*-acetyl-α-D-glucosyl-1-bromide were added under cooling. The mixt. was stirred at 0°C for 3 h, then filtered and the residue extracted several times with 20 ml EtOH each. The combined filtrates were evapd, the residue taken up in EtOAc–H_2O (1:1), the organic phase sepd and the aq. phase extracted ×3 with a total of 150 ml EtOAc. The combined EtOAc frs were dried over Na_2SO_4 and the solvent evapd yielding 259 mg (0.33 mmol) (*S*)-[1-^{13}C]2,7-dibenzyl-4′-β-D-tetra-*O*-acetylglucosylcoclaurine.

An aliquot (154 mg, 0.19 mmol) of the above product was dissolved in 15 ml MeOH, combined with 22 mg (0.41 mmol) NaOMe (Aldrich) and the mixt. stirred for 4 h at room temperature. Subsequently, 10 ml H_2O was added, the soln neutralized with 0.2 ml diluted HCl and the solvent evapd. The residue was sepd by TLC in solvent system EtOAc–EtOH–NH_3 (1:1:0.1) and the substance showing R_f 0.45 eluted with MeOH yielding 88 mg (0.14 mmol) (*S*)-[1-^{13}C]2,7-dibenzyl-4′-β-D-glucosylcoclaurine.

The above product (88 mg, 0.14 mmol) was diluted in 10 ml EtOH, 20 mg Pd/C catalyst (10%, Fluka) was added and the mixt. stirred under H_2 for 4 h at room temperature. The reaction was monitored by TLC ($CHCl_3$-Me_2CO-diethylamine, 5:4:1). Subsequently, the catalyst was sepd by filtration, washed with EtOH, the filtrates combined and evapd. The residue was dissolved in a small amount of EtOH and (*S*)-[1-^{13}C]4′-β-D-glucosylcoclaurine (**4b**) ppt. with EtOAc at a yield of 64% (40 mg, 0.09 mmol). ^{1}H NMR (CD_3OD): δ; 2.68–3.36 (6H, H-3, H-4, H-9), 3.40 (1H, *m*, H-5″), 3.63–3.70 (H-2″, H-3″, H-4″), 3.79 (3H, *s*, OMe), 3.86 (2H, *m*, H-6″), 3.98 (1H, *d*, *J* = 132 Hz, H-1), 4.86 (1H, *d*, *J* = 7.5 Hz, H-1″), 6.54 (1H, *s*, H-5), 6.79 (1H, *s*, H-8), 6.86 (2H, *d*, *J* = 8.1 Hz, H-2′, H-6′), 6.95 (2H, *d*, *J* = 8.1 Hz, H-3′, H-5′). ^{13}C NMR (CD_3OD): δ; 29.5 (C-4), 40.2 (C-3), 42.0 (C-9, $^{1}J_{C\text{-}9/C\text{-}1}$ = 36.8 Hz), 55.8 (4′-OMe), 56.9 (C-1), 61.3 (C-6″), 69.3 (C-4″), 73.3 (C-2″), 75.9 (C-5″), 76.1 (C-3″), 100.1 (C-1″), 111.3 (C-8), 114.4 (C-5), 118.7 (C-3′/C-5′), 125.3 (C-4a), 129.5 (C-8a, $^{1}J_{C\text{-}8a/C\text{-}1}$ = 44.7 Hz), 131.9 (C-2′/C-6′), 134.8 (C-1′), 144.7 (C-7), 146.4 (C-6), 157.0 (C-4′).

4.6. Application experiments

Feeding of whole *E. crista-galli* plants was performed using the cotton wick method according to Barton et al. (1968) for 5–7 days.

For application experiments with leaf material, leaves were dissected and immediately dipped into a 0.01 mM aq. soln (0.5 μCi) of the corresponding putative precursor. When the leaves had taken up the radioactive soln, H_2O was added and the plant material further incubated at 18–20°C and 60–65% humidity for a total of 5–7 days.

Feeding experiments with sliced plant material were conducted as follows: 0.5 μCi of the putative precursor in 0.01 or 0.05 mM concentration (0.5 ml) were incubated together with the sliced plant material (ca. 3–4 mm width) in a well of a sealed multiwell plate (24 × 1 ml wells, Nunc) for 24 h at room temperature in continuous light (650 lx) under shaking at 150 strokes min^{-1}.

In feeding experiments with ^{13}C-labelled precursors the labelled compounds were dissolved in a concentration of 0.1 mM without adding unlabelled material. The incorporation rate into erythraline (**1**) after application of ^{13}C-labelled precursors was determined by comparing EI-MS spectra of unlabelled and labelled erythraline (**1**).

For subsequent analysis, the plant material was removed from the incubation mixt., cut into small pieces, if necessary, and extracted ×2 in a total of 20 ml 80% MeOH for 1.5 h. The combined extracts were evapd and the residue subjected to TLC on Polygram Sil G/UV_{254} with $CHCl_3$–MeOH–NH_3 (90:9:1) as solvent system.The products sepd were localized on the plates by scanning the radioactivity.

4.7. Isolation of alkaloids

The alkaloids were isolated with a modified method according to Folkers and Koniuszy (1949). For this purpose, the plant material was extracted ×2 with 80% MeOH for 1–1.5 h at 70°C. The extracts were combined and evapd, the remaining aq. phase acidified with 2 N H_2SO_4 and incubated for a further 1 h at 60°C to cleave any possibly existing alkaloid glycosides. Subsequently, the soln was extracted ×3 with a total of 300 ml EtOAc or CH_2Cl_2 to remove acidic and neutral compounds. Then, the pH of the remaining mixt. was adjusted to 9–10 with conc. NH_3 and the alkaloids extracted ×3 with a total of 300 ml EtOAc. The alkaloids contained in that extract were

separated by TLC in solvent system $CHCl_3$–MeOH–NH_3 (90:9:1), rechromatographed, if necessary, and subjected to physical measurements.

Acknowledgements

The authors are indebted to Professor Dr. G. Bringmann, Institut für Org. Chemie, Universität Würzburg, Germany for helpful discussions of a possible mechanism for *Erythrina* alkaloid formation. We are grateful to Professor Dr. F. Garbari, Dipartimento di Scienze Botaniche, Universita di Pisa, Pisa, Italy for allowing us to harvest the fruits of *E. crista-galli* and his kind hospitality. Our thanks are due to Dr. J. Page for his linguistic help in the preparation of this manuscript. This work was supported by SFB 369 of the Deutsche Forschungsgemeinschaft, Bonn and by Fonds der Chemischen Industrie.

References

Barrett, A. G. M., Barton, D. H. R., Franckowiak, G., Papaioannou, D., & Widdowson, D. A. (1979). *Journal of the Chemical Society Perkin Transactions I*, 662.

Barton, D. H. R., Boar, R. B., & Widdowson, D. A. (1970a). *Journal of the Chemical Society C*, 1208.

Barton, D. H. R., Boar, R. B., & Widdowson, D. A. (1970b). *Journal of the Chemical Society C*, 1213.

Barton, D. H. R., & Cohen, T. (1957). In *Festschrift Professor Dr. Arthur Stoll* (p. 117). Basel: Birkhäuser.

Barton, D. H. R., Gunatilaka, A. A. L., Letcher, R. M., Lobo, A. M. F. T., & Widdowson, D. A. (1973). *Journal of the Chemical Society Perkin Transactions I*, 874.

Barton, D. H. R., James, R., Kirby, G. W., Turner, D. W., & Widdowson, D. A. (1968). *Journal of the Chemical Society C*, 1529.

Barton, D. H. R., Potter, C. J., & Widdowson, D. A. (1974). *Journal of the Chemical Society Perkin Transactions I*, 346.

Bhakuni, D. S. & Singh, A. N., (1978). *Journal of the Chemical Society Perkin Transactions I*, 618.

Corey, E. J., Gras, J.-L., & Ulrich, P. (1976). *Tetrahedron Letters, 11*, 809.

Folkers, K., & Koniuszy, F. (1949). *Journal of the American Chemical Society*, *62*, 1677.

Franck, B., & Teetz, V. (1971). *Angewandte Chemie*, *83*, 409.

Gerardy, R., & Zenk, M. H. (1992). *Phytochemistry*, *32*, 79.

Ghosal, S., Majumdar, S. R., & Chakraborti, A. (1971). *Australian Journal of Chemistry*, *24*, 2733.

Greshoff, M. (1890). *Chemische Berichte*, *23*, 3537.

Hewgill, F. R., & Pass, M. C. (1985). *Australian Journal of Chemistry*, *38*, 537.

Hider, R. C., Walkinshaw, M. D., & Saenger, W. (1986). *European Journal of Medicinal Chemistry-Chemical Therapy*, *21*, 231.

Ito, K., Haruna, M., Jinno, Y., & Furukawa, H. (1976). *Chemical and Pharmaceutical Bulletin of Japan*, *24*, 52.

Kratzl, K., & Billek, G. (1951). *Monatshefte für Chemie*, *82*, 568.

Kupchan, S. M., Kim, C. K., & Lynn, J.-T. (1976). *Journal of the Chemical Society Chemical Communications*, 86.

Kutchan, T. M. (1998). In G. A. Cordell (p. 257). *Alkaloids, 50*. San Diego: Academic Press.

Maier, U. H., & Zenk, M. H. (1997). *Journal of the Chemical Society Chemical Communications*, 2313.

Roth, L., Daunderer, M., & Kormann, K. (1994). *Giftpflanzen-Pflanzengifte*. Landsberg/Lech: ecomed-Verlagsgesellschaft.

Stadler, R., & Zenk, M. H. (1990). *Liebigs Annalen der Chemie*, 555.

Stadler, R., & Zenk, M. H. (1993). *Journal of Biological Chemistry*, *268*, 823.

Zenk, M. H. (1995). In B. T. Golding, R. J. Griffin, & H. Maskill, *Organic reactivity: physical and biological aspects* (p. 89). RSC: London.