ISOVINCOSIDE (STRICTOSIDINE), THE KEY INTERMEDIATE IN THE ENZYMATIC FORMATION OF INDOLE ALKALOIDS

J. STÖCKIGT and M. H. ZENK
Lehrstuhl für Pflanzenphysiologie, Ruhr-Universität Bochum, D4630 Bochum, FRG

Received 22 March 1977

1. Introduction

Indole alkaloids are among the most complex and diverse low molecular natural products. Their biosynthesis has been investigated by precursor feeding experiments in vivo [1–4] using differentiated *Apocynaceae* plants. As a result of these studies synthetic vincoside, which was originally assigned the 3 α (*S*) stereochemistry, was found to be incorporated into serpentine and other *Corynanthe* type alkaloids in good yield while the incorporation of isovincoside with opposite configuration at C-3 was lower by a factor of 10^3 [5]. In subsequent experiments the crucial problem of the configuration at C-3 of the alkaloidal secoiridoid glucoside was solved and three independent groups reported vincoside to possess a C-3 (*R*) β hydrogen [6–8]. The revision of the absolute C-3 hydrogen stereochemistry to 3 (*R*) β was secured recently by X-ray diffraction experiments [9]. The problem of inversion arose in that vincoside, the C-3 (*R*) β epimer, is considered the biosynthetic precursor of the *Corynanthe*-type alkaloids with 3 α (*S*) stereochemistry.

Recently, the limitations of in vivo experiments for the elucidation of alkaloid biosynthesis were overcome when Scott and Lee [10] obtained a crude cell-free system from callus tissue of *Catharanthus roseus* which was capable of synthesizing geissoschizine and ajmalicine. These experiments have been extended by us and it was shown that a cell-free preparation from fermenter-grown [11] *C. roseus* cells synthesized ajmalicine, 19-epiajmalicine and tetrahydroalstonine from tryptamine and secologanin in the presence of either NADPH or NADH [12]. Furthermore, an immediate precursor of these alkaloids was found to accumulate in the absence of reduced pyridine nucleotides which was identified as 20,21-didehydro-ajmalicine (cathenamine) with 3 α (*S*) configuration [13].

In the present communication we report that using the cell-free system from *Catharanthus* and also an enzyme preparation from *Rhazia stricta* cells we were able to isolate the enzymatically catalyzed primary condensation product between tryptamine and secologanin which proved in both cases to be a single product with 3 α (*S*) configuration. This product is converted by a crude enzyme preparation from *C. roseus* cells to cathenamine and in the presence of reduced nucleotides to ajmalicine, 19-epiajmalicine and tetrahydroalstonine. In contrast to previous assumptions [5,8], the key stone in the intricate pathway which leads to the structurally diverse indole alkaloids is isovincoside (strictosidine) (3 α (*S*)) and not vincoside (3 β (*R*)).

2. Materials and methods

Catharanthus roseus cells were grown as previously reported [11]. *Rhazia stricta* callus was initiated from surface sterilized seeds (collected in Pakistan) on 4 X medium [14]. After 16 months of subculturing an evenly growing cell suspension was achieved. *Daucus carota* and *Nicotiana tabacum* were standard cultures of this laboratory and grown also in 4 X medium.

Crude enzyme preparations were obtained as previously described [12] but an ammonium sulfate cut of 35–50% was used and the enzyme preparation

was subsequently dialysed for 2 h against 0.1 M borate buffer, pH 7.6, containing 20 mM β-mercaptoethanol.

The reaction mixture was spotted onto thin-layer plates SIL G/UV_{254} (Macherey and Nagel) and developed in the solvent system I (acetone/methanol/diethylamine, 70:20:10). The main radioactive band (R_F 0.53) was eluted with MeOH, the compound acetylated [5] mixed with standards and used for chromatographic identification in four different solvent systems:II (benzene/ethylacetate, 1:2); III (ethylacetate/*n*-hexane, 7.5:5); IV (benzene/acetone/light petrol (40–60°C) 5:2:3); V (ethylformiate/ether/*n*-hexane, 5:1:1).

The pentaacetate of the glucoalkaloids were further identified by formation of *N*-acetylderivatives [5] and determination of their specific activities. *N*-Acetylvincoside was recrystallized to obtain constant specific activity (from EtOH and EtOH/H_2O, 1:0.5) and *N*-acetylisovincoside, was purified by chromatography in solvent VI ($CHCl_3$/MeOH, 5:1).

For CD determination, a large scale enzyme incubation mixture was taken to dryness in vacuo and acetylated. Penta-acetyl-isovincoside was isolated using preparative layer (1.5 mm) chromatography and solvent system VII (benzene/ethylacetate, 1:1.5). The derivative was subsequently rechromatographed using solvent system II and eluted using a mixture of $CHCl_3$/MeOH (1:1). CD determinations were performed in acetonitrile. The isolated compounds were subjected to masspectroscopy using a VARIAN Mat III at an electron energy of 80 eV. [2-^{14}C]tryptamine was purchased from NEN, [G-^{3}H]tryptamine from Amersham, secologanin was isolated from *Lonicera* sp. [15], all biochemicals were from Boehringer, Mannheim.

3. Results

The nonenzymatic condensation of tryptamine with secologanin to yield a mixture of vincoside and isovincoside has been described in detail [5]. In the course of indole alkaloid biosynthesis the glucose moiety of vincoside is split off by a glucosidase within the plant. D-δ-gluconolactone is known as an inhibitor of several β-glucosidases. Therefore it was hoped that inclusion of the lactone in the enzyme preparation would lead to the accumulation of the intermediate alkaloidal glucoside. Indeed it was found that 100 mM gluconolactone inhibited the over all reaction by more than 95% [16].

When [2-^{14}C]tryptamine (125 nmol; 0.2 μCi) was incubated with secologanin (625 nmol) the enzyme mixture from *Catharanthus* (0.1 mg) and D-δ-gluconolactone (50 μmol) at pH 6.1, for 120 min, chromatographic analysis of the reaction mixture showed the formation of a condensation product in up to 50% yield. In subsequent experiments it was found, that the gluconolactone could be omitted when the reaction was conducted at the pH optimum of the product accumulation, at pH 4.1, in citrate buffer. The reaction proceeds only in the presence of functional enzyme (fig.1A) and is strictly enzyme-dependent.

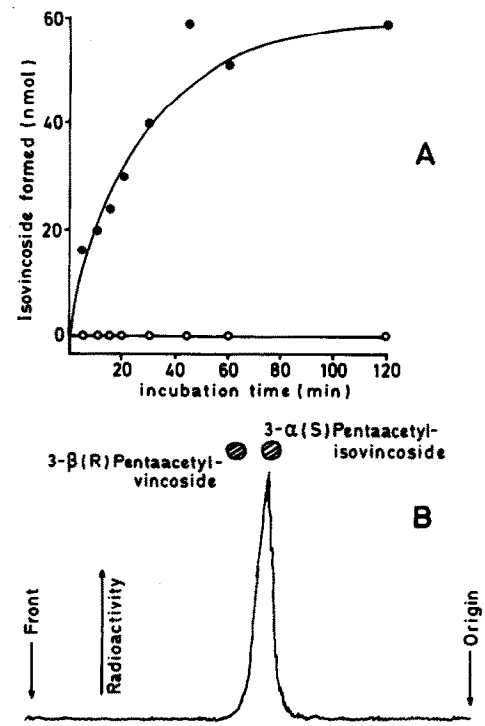

Fig.1. Enzymatic synthesis of isovincoside (strictosidine) and its chromatographic identification as pentaacetate. (A) Reaction mixtures contained in final vol. 0.5 ml: 50 μmol potassium phosphate, pH 7.5, 125 nmol [2-^{14}C]tryptamine (0.2 μCi), 625 nmol secologanin, 50 μmol D-δ-gluconolactone, and 0.1 mg protein (●●●) or either no or heat denatured protein (ooo). (B) Thin-layer chromatography in solvent system II of enzymatically formed isovincoside as the pentaacetate with authentic penta-acetylvincoside and -isovincoside.

For the identification of the condensation product it was chromatographed in solvent system I which showed the total radioactivity to be associated with the position of isovincoside (R_F 0.53). Synthetic vincoside under these conditions (alkaline solvent system) is converted to vincoside lactam (R_F 0.44) which was identified as its tetraacetate [5] (M^+ 666 m/e). The observed stability of the labelled compound at room temperature under alkaline conditions is in accordance with the properties previously found for isovincoside [5].

Furthermore, the labelled compound was acetylated with acetic anhydride and the product identified by mass spectrometry as the pentaacetate (M^+ 740 m/e). The fragmentation pattern was identical to that of the authentic isomers. Thin-layer chromatography in 4 different solvent systems (II–V) of the labelled pentaacetate showed an exact correspondence of the unknown pentaacetate with an authentic sample of penta-acetylisovincoside (fig.1B). In all solvent systems used, penta-acetylisovincoside moved slower than penta-acetylvincoside. The structure of the enzymatically-formed labelled intermediate was further analysed by derivatisation. To a mixture of vincoside and isovincoside, obtained by chemical condensation of 300 mg secologanin with 150 mg tryptamine hydrochloride according to the standard procedure [5], an incubation mixture containing 63 nmol (0.25 μCi) of the enzymatically-formed alkaloidal glucoside was added and the mixture immediately acetylated.

The acetylation products were separated by preparative thin-layer chromatography in solvent VII and yielded 132 μmol penta-acetylvincoside (spec.act. 25 dpm/μmol) and 71 μmol penta-acetylisovincoside (spec.act. 2.62×10^3 dpm/μmol). Both pentaacetates were *O*-deacetylated [5] to yield *N*-acetylvincoside (114 μmol) (M^+ 572 m/e) as needles (EtOH) which was rigorously purified (m.p. 180–181.5°C) to spec.act. 3 dpm/μmol. While *N*-acetylisovincoside 59 μmol (M^+ 572 m/e) gave a foam with spec.act. 2.50×10^3 dpm/μmol. When other derivatives of both epimers such as the lactams, 18,19-dihydro-pentaacetates, or the corresponding dihydro-*N*-acetyl compounds were prepared, label was found exclusively in the isovincoside derivatives.

Final proof of the enzymatic product to be isovincoside came from CD-comparison of authentic vincoside and isovincoside pentaacetates with a purified sample (6.85 mg) of the unknown product isolated as pentaacetate from a 37 ml enzyme (*Catharanthus*) incubation under the above conditions (pH 4.1, 120 min, 28°C). A total of 13.5 μmol (60%) of the intermediate had been formed under these conditions. The CD-curves of the acetylated product of the enzymatic reaction and of penta-acetylisovincoside were, indeed, absolutely identical.

It has been shown by the experiments reported above, that the enzymatically-catalyzed condensation of tryptamine and secologanin yields isovincoside. Proof still has to be presented that this compound is a true intermediate in the enzymic formation of *Corynanthe* alkaloids. Enzymatically-formed isovincoside (0.8 nmol, 0.8 μCi) generally labelled with ^3H in the tryptamine derived portion of the molecule was incubated with NADPH (1.0 μmol) in the presence of 0.1 M buffer, pH 7.5 and 0.5 mg protein from *C. roseus* in total vol. 0.5 ml. After 60 min incubation at 28°C, 0.32 nmol of ajmalicine isomers were formed (46% ajmalicine, 36% 19-epiajmalicine, 18% tetrahydroalstonine). In the absence of NADPH cathenamine was formed in 37% yield under these conditions. In the control experiments using the same mixture but heat denatured enzyme no reaction occurred in both cases.

As a final point, the identity of alkaloidal glucoside formed from secologanin and tryptamine by cell-free extracts of suspension cells of *Catharanthus*, with the product formed by enzymes from *Rhazia stricta*, was investigated. The latter plant is known to accumulate isovincoside (strictosidine) [17]. Crude enzyme preparations from vigorously growing *Rhazia stricta* cells were incubated with 125 nmol [^{14}C]tryptamine (0.2 μCi) and 625 nmol secologanin in the presence of 0.1 M phosphate buffer, pH 7.5, 0.1 M gluconolactone and 1.36 mg protein. After 90 min incubation, 75 nmol of the glucoalkaloid had been formed. The labelled product was identified by chromatography (solvent I) and after acetylation as penta-acetylisovincoside (solvents II–V). The isovincoside thus formed by catalysis of the *Rhazia stricta* enzyme was isolated and purified as above and subsequently 16 nmol (5.68×10^4 dpm) were incubated with 2 μmol NADPH in the presence of 0.1 M phosphate buffer, pH 7.5 and 1.1 mg *Catharanthus* enzyme (vol. 0.5 ml, incubation 120 min, 28°C) which gave a 14% conversion into all three ajmalicine isomers.

Similar protein fractions from *Daucus carota* or *Nicotiana tabacum* or heat denatured protein from *Catharanthus* did not catalyze the formation of the isovincoside. It can therefore be stated that both plants, *Catharanthus* and *Rhazia*, which had previously been assumed to synthesize mainly either vincoside or isovincoside, do in fact enzymatically produce only one epimer, namely the C-3 (*S*) α isovincoside (strictosidine).

4. Discussion

Previously it has been regarded as an intriguing point which emerged from the biosynthetic studies on monoterpenoid indole alkaloids, that for *Corynanthe* type alkaloids with 3 α (*S*) stereochemistry, the exclusive precursor was assumed to be vincoside with 3 β (*R*) configuration and that the inversion on C-3 occurred with retention of the hydrogen atom [18]. Chemical model reactions for the epimerisation have been worked out [19] which provided satisfactory analogies for the assumed biosynthetic events.

On the basis of the results presented in this paper these assumptions are unnecessary. It was clearly demonstrated that the product of the enzymatically catalyzed reaction between secologanin and tryptamine is exclusively isovincoside (strictosidine) with 3 α (*S*) stereochemistry. It is this configuration which is needed for the biosynthetic formation of the monoterpenoid indole alkaloids. The enzymatically formed isovincoside studied here has all chemical and physical properties which have previously been reported for synthetic isovincoside [5]. This product is formed when either an enzyme from *Catharanthus* is used, a plant which has been assumed to form vincoside as biosynthetic intermediate [5], or from *Rhazia stricta* which has been known to be a source of strictosidine (isovincoside) [17]. The possibility that inversion at C-3 occurs during the isolation of the enzymatically formed product can be excluded since acetylation, which does not produce inversion in this glucoalkaloid series [5] yielded exclusively isovincoside pentaacetate. The isovincoside thus formed is transformed to cathenamine in the absence of reduced pyridine nucleotides and in their presence to ajmalicine, 19-epiajmalicine and tetrahydroalstonine by cell free extracts from *Catharanthus*. We can not yet explain, however, the finding [5] which is based on feeding experiments using differentiated *Catharanthus* plants, that vincoside is efficiently incorporated into monoterpenoid indole alkaloids while isovincoside (strictosidine) is not involved in these in vivo biosyntheses.

Based on the above and our previous [12,13] work using cell free systems from *Catharanthus* suspension cells we are now able to formulate the biosynthesis of monoterpenoid indole alkaloids of the ajmalicine group as it is depicted in fig.2.

Because of the previous confusion with regard to the stereochemistry of C-3 of vincoside and isovincoside [5–8] and the fact presented here that isovincoside rather than vincoside is the true intermediate in indole alkaloid biosynthesis we propose that the name strictosidine (3 α (*S*)) should be used for this

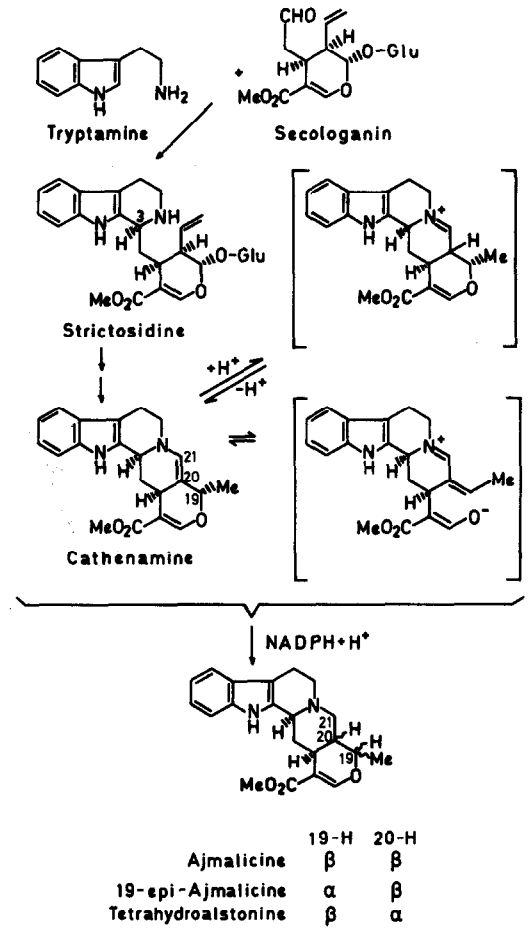

Fig.2. Biosynthetic pathway of *Corynanthe* alkaloid formation in *Catharanthus roseus*.

key intermediate. This name proposed by G. N. Smith for the 3 α (*S*) epimer deserves priority [6,17].

Acknowledgements

Our thanks are due to Miss B. Ries for excellent technical assistance, to Professor G. Snatzke and Dr F. Snatzke for measuring the CD curves, to Professor A. Battersby, Cambridge, for a sample of isovincoside, to Dr C. R. Hutchinson, Madison, for samples of the pentaacetates of both vincoside and isovincoside and to the Bundesminister für Forschung und Technologie, Bonn, for financial support.

References

[1] Escher, S., Loew, P. and Arigoni, D. (1970) Chem. Commun. 823–825.
[2] Battersby, A. R. (1972) Acc. Chem. Res. 5, 148–154.
[3] Scott, A. I., Reichardt, P. B., Slaytor, M. B. and Sweeney, J. G. (1971) Bioorg. Chem. 1, 157–173.
[4] Parry, R. J. (1975) in: The *Catharanthus* Alkaloids (Taylor, W. I. and Farnsworth, N. R. eds) Marcel Dekker, Inc. New York, p. 141.
[5] Battersby, A. R., Burnett, A. R. and Parsons, P. G. (1969) J. Chem. Soc. (C), 1193–1200.
[6] DeSilva, K. T. D., Smith, G. N. and Warren, K. E. H. (1971) Chem. Commun. 905–907.
[7] Blackstock, W. P., Brown, R. T. and Lee, G. K. (1971) Chem. Commun. 910–911.
[8] Kennard, O., Roberts, P. J., Isaacs, N. W., Allen, F. H., Motherwell, W. D. S., Gibson, K. H. and Battersby, A. R. (1971) Chem. Commun. 899–900.
[9] Mattes, K. C., Hutchinson, C. R., Springer, J. P. and Clardy, J. (1975) J. Amer. Chem. Soc. 97, 6270–6271.
[10] Scott, A. I. and Lee, S.-L. (1975) J. Amer. Chem. Soc. 97, 6906–6908.
[11] Zenk, M. H., El-Shagi, H., Arens, H., Stöckigt, J., Weiler, E. W. and Deus, B. (1977) in: Plant cell cultures and its biotechnological application, Springer-Verlag, Berlin, Heidelberg, New York, in press.
[12] Stöckigt, J., Treimer, J. and Zenk, M. H. (1976) FEBS Letters 70, 267–270.
[13] Stöckigt, J., Husson, H. P., Kan-Fan, C. and Zenk, M. H. (1977) Chem. Commun. in press.
[14] Gamborg, O. L., Miller, R. A. and Ojima, K. (1968) Exptl. Cell Res. 50, 151–158.
[15] Souzu, I. and Mitsuhashi, H. (1970) Tetrahedron Lett. 191–192.
[16] Treimer, J. and Zenk, M. H. unpublished results.
[17] Smith, G. N. (1968) Chem. Commun. 912–914.
[18] Staunton, J. (1972) in: Chemical society specialist periodical report, the alkaloids, Vol. 2 (Saxton, J. E. ed) p. 1, The Chemical Society, London.
[19] Brown, R. T., Chapple, C. L., Platt, R. and Spencer, H. (1974) Chem. Commun. 929–930.