Phytochemistry, 1975, Vol. 14, pp. 2407–2409. Pergamon Press. Printed in England.

CONVERSION OF SQUALENE-2(3) EPOXIDE BY ENZYMES OF *ALNUS GLUTINOSA*

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(*Received* 31 *January* 1975)

Key Word Index—*Alnus glutinosa*; Betulaceae; squalene-2(3) epoxide; squalene-2(3) epoxide cyclases; cycloartenol; biosynthesis; backbone rearrangement; cell-free system.

Abstract—The incubation of [1-^{14}C]-squalene-2(3)epoxide ([1-^{14}C]-SO) with a cell-free extract of *Alnus glutinosa* gave only cycloartenol in 1% yield. The effects of pH, detergent (Triton) and enzyme/substrate ratio upon cyclase activity were studied in order to determine the optimal conditions for the enzymatic conversion of [1-^{14}C]-SO to cycloartenol.

INTRODUCTION

Several examples of enzymatic cyclization of [1-^{14}C]-squalene-2(3)epoxide (SO) to triterpenoids by cell-free extracts of both animals and plants are known and it is clear from a comparison of the structures of many triterpenes that several types of cyclase must exist [1], but the formation of any triterpene which results both from biosynthetic backbone rearrangement (BBR) and which gives a backbone rearrangement in acidic medium (BRA) [2] has not been described.

Alnus glutinosa is known to contain considerable quantities of glutinone [3, 4], a triterpene which satisfies the above two conditions; thus, we have attempted to label glutinone by enzymatic conversion from [1-^{14}C]-SO using cell-free extracts of this plant. Under our experimental conditions, neither labelled glutinone nor any other triterpene of the same family was obtained; the only labelled triterpene we could isolate was cycloartenol.

RESULTS

When [1-^{14}C]-SO was incubated with a cell-free extract of *A. glutinosa* two radiolabelled fractions F_1 and F_2 were isolated which were less polar and more polar respectively than [1-^{14}C]-SO. (In the following M_1 and M_2 refer respectively to the total radioactivity of F_1 and F_2, M'_1 and M'_2 refer to the radioactivity of the same fractions F'_1 and F'_2 relative to the control.) F_1 and F'_1 had the same radioactivity and were identical on preparative GLC; F_1, which probably corresponds to a degradation product of [1-^{14}C]-SO, was not further studied. Identical aliquots (a and a') of F_2 and F'_2 were analysed by preparative GLC, 5 min fractions were collected and their radioactivity measured; the distribution of radioactivity, given in Fig. 1 shows a conversion only for the fraction F_2. The R_t of the conversion products (peak C, Fig. 1) is the same as those of glutinol, cycloartenol or taraxerol which are not separated by GLC. However, continuous TLC (15 hr; eluant $CHCl_3$, adsorbent Si gel + 10% $AgNO_3$) gave a successful separation: using 3β-glutinol as reference (R_f = 1), the R_f's of taraxerol, cycloartenol and 3α-glutinol were 0·9; 0·8 and 0·5 respectively. Under these conditions, fraction C (Fig. 1) had an R_f = 0·8 on TLC which corresponds therefore to cycloartenol. Furthermore, after dilution of 5000 dpm of radioactive cycloartenol fraction isolated from the incubation with 30 mg of non-radioactive cycloartenol, this

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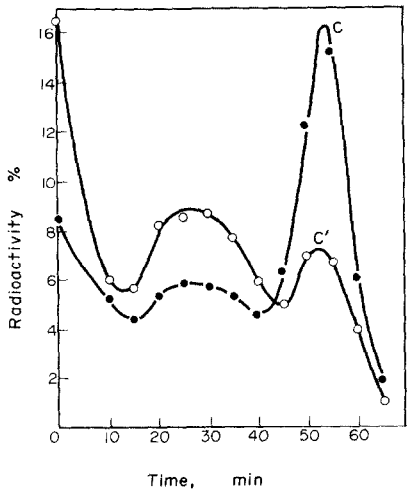

Fig. 1. Distribution of radioactivity (%) in fractions F-2 and F'2; O——O fraction F-2; ●——● fraction F-'2. The retention time of product (peak C) was 53 min.

mixture was recrystallized from $CHCl_3$–MeOH (6:4) to constant sp. act. (90 dpm/mg).

Optimal conditions for enzymatic activity

Activity was evaluated from the percentage value of the apparent conversion (% A.C.): % A.C. = %C. M_2/a − % C' M'_2/a' (C and C' are percentages of the radioactivity in Fig. 1). The effects of pH, detergent (Triton) and enzyme:substrate ratio upon cyclase activity were studied in order to determine the optimal conditions for the enzymatic conversion of [1-^{14}C]-SO to cycloartenol. The optimum pH of the enzyme was determined by adjusting the phosphate components of buffer A (see Experimental) between 6·8 and 7·3. Both the detergent:substrate ratio (=1:1) and the volume of the enzyme preparation (2 ml) were constant during this study. The optimum pH of this cyclase preparation was 7·1. It was found that Triton X-100 was superior to Tween-80 for the solubilization of the substrate. The optimum Triton X-100:substrate ratio is 1:1 (w/w), % apparent conversion 4·1. Throughout this experiment the pH of the solution was 7·2 and the volume of the enzyme preparation was 2 ml. At pH 7·2 and Triton:SO = 1:1, we also studied the effects of adding increasing amounts of enzyme preparation to 4 μg [1-^{14}C]-SO. The results showed that the optimum volume was 2 ml of enzyme preparation per 4 μg of SO (% apparent conversion 2·4).

DISCUSSION

This work described a new example of enzymatic conversion of [1-^{14}C]-SO to cycloartenol. It is interesting that under our experimental conditions, only labelled cycloartenol was isolated: no labelling of either glutinol or glutinone was observed. The seedlings of *A. glutinosa* used in our experiments contained sitosterol 20%, glutinol 2%, lupeol 5%, cycloartenol 5% and taraxerol 3% (% unsaponifiable lipid fraction by GLC). This conversion contrasts with results obtained from studies of the crude triterpene cyclases of *Ononis spinosa* and *Pisum sativum* (Dean, unpublished observations) where different cyclases may be identified either with different enzyme and substrate concentration [5] or at different stages of seedling development (*P. sativum*).

The absence of labelled glutinol in *A. glutinosa* leads us to consider various possible explanations: (i) SO may not be a precursor of glutinone (or glutinol); (ii) the specific enzymatic system for the conversion of [1-^{14}C]-SO to glutinone (or glutinol) may genuinely be lacking in the plant tested, or may not be stable after cell rupture and may only appear to be absent in the cell-free extract; (iii) activator(s) of the cyclase or synergic biological factor(s) (for instance coupling of the cyclization reaction with an exergonic reaction) are required.

The first hypothesis is very improbable, but cannot be ruled out. The second possibility seems unlikely since glutinol was found in abundance not only in the 3-month-old plants used in the experiments, but also in younger seedlings which did not give labelled glutinone or glutinol when incubated with [1-^{14}C]-SO. Furthermore, neither rapid degradation nor intrinsic instability has been observed with other cyclases: indeed, these enzymes seem to be relatively stable, at least in crude extracts.

Hypothesis (iii) seems more plausible since the possibility of a bioenergetic coupling is supported by the observation that no triterpene with high potential energy (Group 1, Table 1) has been obtained from SO in cell-free media. This may not be merely due to chance and it supports the idea that SO cyclization to triterpenes of this Group 1 involves, in addition to the cyclase, other factors to be determined.

Table 1. Classification of principle groups of natural triterpenoids involving a biosynthetic backbone rearrangement (BBR) or a backbone rearrangement in acidic medium (BRA)

1 BBR + BRA		2 Only BBR	3 Only BRA	4 No BR
Taraxerane, Multifluorane, Glutinane, Friedelane	Friedooleane	Lanostane, Cycloartane	Protostane, Lupane, Neogammacerane, 21-Isoncogammacerane	Oleane, Ursane, Gammacerane, Taraxastane
Bauerenane	Friedoursane			
Fernane, Filicane, Adianane	Friedoneogammacerane			
Euphane	Epilanostane			

BR = Backbone rearrangement.

EXPERIMENTAL

NMR spectra were recorded at 60 MHz using TMS as an internal standard. mp's are uncorrected. Radioactivity was measured by liquid scintillation counting in 15 ml of the following mixture: PPO 3 g; POPOP 0·1 g; toluene 1 l. Preparative GLC was performed on an XE60 column at 230° using N_2 at 10 ml/min. Optical rotations were measured in *ca* 1% solns in $CHCl_3$. TLC was carried out on Si gel F_{254} plates. Triton X-100 was used as an emulsifying agent. The Pi buffer A (pH 7·2) contained KH_2PO_4 (0·1 M), K_2HPO_4 (0·2 M), cysteine (0·05 M), sucrose (0·2 M), ascorbate (0·05 M), PVP (2%) and KCl (0·15 M) in double dist. H_2O. Cycloartenol was extracted, after saponification, from *Pastacia lentiscus* [6] mp = 112°; $[\alpha]_D^{20} = +51 \pm 3$ ($CHCl_3$); $M^+ = 426$ ($C_{30}H_{50}O$) *m/e* 408, 286. The NMR and IR spectra were identical with those described in the literature [6, 7]. [1-^{14}C]-Squalene-2(3)-epoxide, sp. act. 23 mCi/mmol, was prepared by a previously described method [8].

Preparation of enzyme. These expts were carried out as described by Rowan *et al.* [9]. Both roots and leaves of 3-month-old seedlings of *A. glutinosa* were used as a source of enzyme. About 15 g of tissue were pulverized with a pestle and mortar under liquid N_2. The frozen powder was stirred into buffer A (30 ml) at 4°. The homogenate was strained through gauze and filtrate centrifuged at 3500 *g* for 20 min. The pellet was discarded. All manipulations were carried out at 0–4°.

Incubations. Enzyme preparation (2 ml/tube) and an emulsion of the substrate ([1-^{14}C]-SO, 0·2 μCi/tube) with Triton X-100 (detergent:substrate ratio = 1:1 (w/w)) prepared as described in Ref. [10] were incubated with shaking, under N_2 at 25° for 16 hr. Control incubations were carried out with boiled enzyme preparations (100° for 15 min). The reaction was stopped by adding an equal vol. of 2 M KOH in MeOH. This mixture was heated with shaking at 50° for 3 hr. The soln was extracted using a Vortex mixer, with pentane (2 × 5 ml), hexane (2 × 5 ml) and Et_2O (5 ml). Each extraction was followed by centrifugation and separation of the organic layer. The organic layers were combined, dried and evaporated to dryness, *in vacuo*. The products were separated by TLC on Si gel hexane–EtOAc, 5:1). Unreacted [1-^{14}C]-SO (110000 cpm = 80%) and two radioactive fractions: F_1 less polar than SO (5000 cpm = 4%) and F_2 more polar than SO (20000 cpm = 16%) were separated.

Acknowledgements—We wish to thank Mr. J. K. Hulme of the Liverpool University Botanic Gardens for his willing assistance with the supply of seedlings, and Professor Ourisson for his gift of cycloartenol. This work was partly supported by grants from Royal Society of London and C.N.R.S. in France, which we gratefully acknowledge.

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