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STEREOSPECIFIC ENZYMIC CYCLIZATION OF A SYNTHETIC 2,3-OXIDOSQUALENE ANALOGUE BEARING AN 18Z CARBON-CARBON DOUBLE BOND [25]

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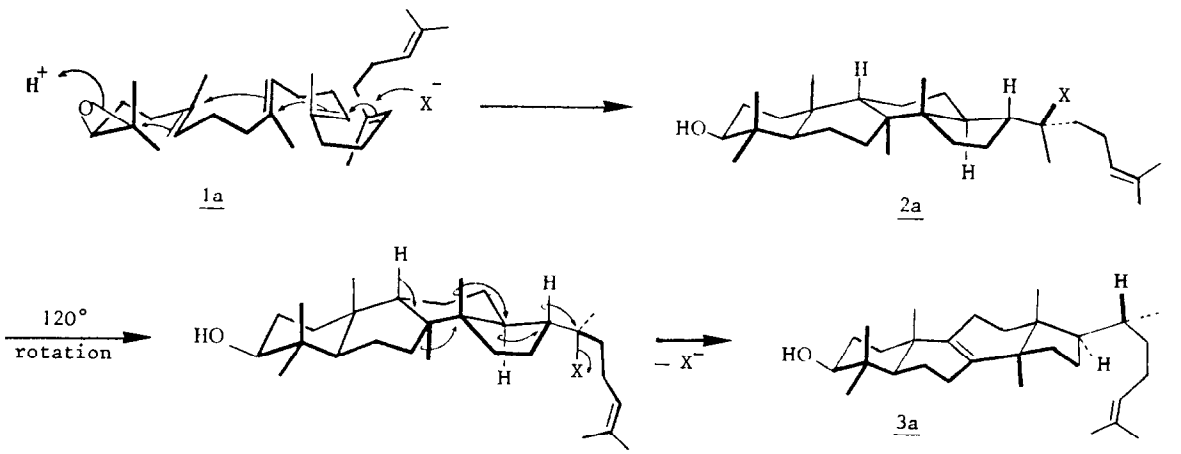
This report discloses the first successful cyclization of a squalene analogue bearing a Δ^{18} Z instead of E C=C bond which leads to a lanosterol analogue possessing the unnatural 20S stereochemistry. The hypotheses of sterol biosynthesis are discussed in the light of these findings.

Since the first hypothesis of Woodward and Bloch[1] involving all trans squalene as the biogenetic precursor of sterols and the brilliant theoretical models constructed independently by the Zurich School[2] and by Stork[3] to explain this spectacular transformation, much work has been devoted to the biosynthesis of lanosterol [4,5] and analogues from squalene, from the corresponding 2,3-oxide, its closest precursor known, and from labelled analogues [4].

However, the problems related to the C-20 carbon atom on the sterol have not been yet clarified[6] although its crucial role on the protosterol 2a was early recognized. The C-20 carbon atom in the protosterol 2a, featured as a classical or a non-classical[2] carbocation, attached to the enzyme[5] or bounded to an exogeneous nucleophile [5] (Scheme I), is thought to initiate, after the appropriate movement about the C-17 - C-20 axis, the 1,2-hydrogen-hydrogen-methyl-methyl migrations leading to lanosterol.

SCHEME I [5]



In order to test if the natural 20R stereochemistry at C-20 in lanosterol 3a is directly related to the stereochemistry of the Δ^{18} carbon-carbon double bond on the starting oxidosqualene, (21-^{14}C)-2,3(RS)-oxido-2,6,10,15,19-pentamethyl-heneicosa-6E,10E,14E,18Z-tetraene 4b, bearing a 18Z instead of the natural 18E carbon-carbon double bond, was stereoselectively (95%) synthesized [7]

and reacted with 2,3-oxidosqualene sterol cyclase. Our preliminary results are described below.

Anaerobic incubation of the labelled oxide 4b (230µg, 3.9 10^6 dpm) with a solution of 2,3-oxidosqualene sterol cyclase [8,9] (6ml) at 20° affords, in addition to unchanged labelled oxide 4b (47%) and polar labelled products[10] (23%), a third radioactive fraction (Fraction A - 60% conversion)[11,12] indistinguishable from lanosterol 3a by tlc (SiO_2 Merck, benzene/ethyl acetate : 97/3, R_f 0.20). *This fraction A clearly contains a biosynthetic product since no radioactivity is found at R_f 0.20 (see above) when denatured (preboiled) 2,3-oxidosqualene sterol cyclase is used.*

Fraction A is acetylated with excess acetic anhydride-pyridine (20°, 12 hr). The resulting product migrates as a single radioactive [12] spot on tlc, identical to lanosteryl acetate 5a and its dihydroderivative 6a (SiO_2 Merck, benzene/ethyl acetate 98/2, two developments R_f 0.70)[12] but is resolved into two distinct radioactive [12] spots on silver nitrate impregnated tlc (SiO_2 - $AgNO_3$, hexane/chloroform 80/20 R_f 0.00[10] and R_f 0.24)[12] which separates 6a (R_f 0.24) and 5a (R_f 0.06). Acetylated Fraction A is purified as shown. This allows the recovery of 60% of the radioactivity used at R_f 0.24 (Fraction B).

Fraction B is in fact a complex mixture of products[13a] as observed by $|GC|^2$ but the radioactivity is mainly located (82%) in a single peak (14-19 min) Sterol Fraction S with Rt similar to that of a slightly resolved mixture of norlanosteryl acetates [7] 8a and 8b possessing respectively *20R* and *20S* stereochemistry [on a 40m x 1mm glass capillary column coated with OV1 phase, column temp. 250°, carrier gas (He), flow rate : 12 ml/min] [14]. The remaining radioactivity (18%) is spread over all the other six fractions collected between 1-14 and 19-180 min.

Careful gas chromatography analysis $|GC|^2$ clearly shows that Fraction S is revealed in two peaks[13b,15], the major one γ (93%) being indistinguishable from that of *20S* norlanosteryl acetate 8b, on three different phases (non polar ones such as (SE.30) or (SE.52) and polar (Superox 4)[16]) which allow clean separation of authentic *20R* and *20S* norlanosteryl acetates 8a and 8b.

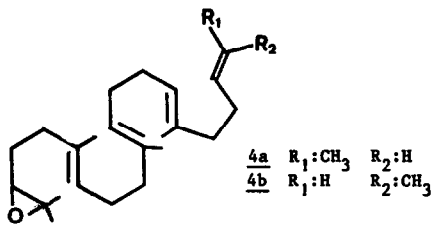
[(1) - on a 30mx0.5 mm glass capillary column statically coated with SE.30, column temp. 283°, carrier gas (He), flow rate : 3ml/min., Rt : γ and 8b : 13.13 min., 8a : 13.45 min.,
(2) - on a 40mx0.25mm glass capillary column statically coated with SE.52, column temp. 240°, carrier gas (He), flow rate : 4.2 ml/min., Rt : γ and 8b : 27.9 min., 8a : 28.7 min.,
(3) - on a 8mx0.5 mm glass capillary column statically coated with Superox 4, column temp. 230°, carrier gas (He), flow rate : 6 ml/min., Rt : γ and 8b : 11.4 min., 8a : 11.8 min.]

Moreover, the mass spectrum of γ on $|GC|^2$ MS discloses the following m/e values : 414 (M^+,1%), 399(21%),243(3%),241(2%) and is close to that of 8b (especially in the m/e 414-241 region).

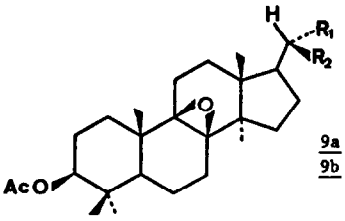
Fraction B is then reacted with excess m-chloroperbenzoic acid in $CHCl_3$ (20%, 4 hr) under conditions which allow the transformation of norlanosteryl acetates 8a and 8b into their corresponding 8,9-epoxides 9a and 9b. The resulting product exhibits only one radioactive spot on tlc (SiO_2, benzene/ethyl acetate, indistinguishable from 9a and 9b). It is purified by this technique and produces Fraction C. Fraction C presents a major peak δ by $|GC|^2$, clearly different from 9a but indistinguishable from the *20S* stereoisomer 9b.

[(1) - on a 30mx0.5 mm, glass capillary column statically coated with SE.30, column temp. 230°, carrier gas (He), flow rate : 5 ml/min., Rt : δ and 9b : 14.54 min., 9a : 15.49 min.,
(2) - on a 55mx0.25mm, glass capillary column statically coated with SE.52, column temp. 250°, carrier gas (He), flow rate : 1.08ml/min., Rt : δ and 9b : 93.6 min., 9a : 96.6 min.]

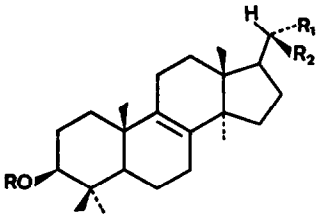
The mass spectrum of δ [$|GC|^2$MS] exhibits the following m/e values : [chemical ionisation (CH_4) 431($M+1^+$,16%), 429(13%), 415(5%), 413(3%), 371(100%), 353(87%), 339, 338, 337] very close to those of 9b.



4a R_1:CH_3 R_2:H
4b R_1:H R_2:CH_3



9a R_1:CH_3 R_2:C_2H_5
9b R_1:C_2H_5 R_2:CH_3



5a	R:Ac	R_1:CH_3	R_2:$(CH_2)_2CH{=}C(CH_3)_2$
6a	R:Ac	R_1:CH_3	R_2:$(CH_2)_3CH(CH_3)_2$
7a	R:H	R_1:CH_3	R_2:C_2H_5
7b	R:H	R_1:C_2H_5	R_2:CH_3
8a	R:Ac	R_1:CH_3	R_2:C_2H_5
8b	R:Ac	R_1:C_2H_5	R_2:CH_3

Furthermore, the radiolabelled compound γ is recovered unchanged [(1) - tlc, SiO_2-$AgNO_3$ - (2) - $|GC|^2$, SE.30 - (3) - transformation to the epoxide δ, above described conditions] when Fraction B is reacted with H_2/Pt (excess in ethyl acetate, 2 hr) under conditions which allow the quantitative reduction of the Δ^{24} double bond in lanosteryl acetate but which do not reduce its highly encumbered Δ^8 double bond.

These preliminary results clearly demonstrate the presence of an hydroxyl group and an encumbered carbon-carbon double bond in the biosynthetic product β which is NOT the NORLANOSTEROL 7a but COULD BE the epiNORLANOSTEROL 7b possessing the 20S stereochemistry.

In order to further support this hypothesis, the radioactive biosynthetic Fraction B and Fraction C were respectively contaminated with synthetic norlanosteryl acetates 8b and 8a, and with 8,9-oxido norlanosteryl acetates 9b and 9a, then recrystallized to constant specific activity. This was readily achieved in experiments involving the *20S* diastereomers 8b [methylene chloride/methanol : 1/4 as solvent, (specific activities in dpm/mg), starting (2000), crystals, respectively : (2000),(1700),(1700); mother liquors : (2450)[17a],(2350),(1950)] and 9b [benzene/methanol[19] : 1/5, (specific activities in dpm/mg); starting (1550), crystals respectively (1600),(1600),(1600); mother liquors (1400)[17b], (1700), (1700)] whereas cocrystallization was not possible in experiments involving the *20R* diastereomers 8a and 9a, the radioactivity being rapidly recovered in the mother liquors [18].

The experimental results presented strongly suggest that the radioactive acetate γ and the synthetic norlanosteryl acetate 8b with the unnatural 20S stereochemistry is one and the same substance and consequently that the biosynthetic product β and 7b are identical.

This report discloses the first successful cyclization of a squalene analogue which does not have the all trans natural stereochemistry. The enzyme having accepted the 18Z-oxidosqualene analogue 4b[20a], a result not predictable from earlier work[4] - must transform it by some strictly stereospecific process in which the geometrical locations of the groups attached to the 18,19-double bond, and not for example their relative size [21], determine the final stereochemistry at C-20 of the steroid. The hypotheses concerning the intermediate(s) formed after cyclization of natural squalene and before the 1,2-shifts should also explain the results just disclosed in order to be valid.

The mechanism requiring a classical carbocation[22] seems unlikely since it is difficult to

imagine how an enzyme would force a rotation to take place in a particular direction (+120° and not -60°) irrespective of the relative size of the two groups at C20 [1a → 3a, 4a → 7a compared to 4b → 7b]. Interestingly however the Cornforth X-group hypothesis[5] (Scheme I) can explain both results since the acceptance of the X-group[20b] at C-20 fixes the stereochemistry there; the conformation for the rearrangement is also unique and can be reached by free rotation in either direction.

Our results disclose another example of the stereoselectivity related to enzymic transformations, a concept so remarkably developed inter alia by Cornforth[23] and Arigoni[24].

The authors sincerely thank Dr.Cornforth for his helpful comment and for the fruitful discussions.

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8. A phosphate buffered microsomal solution (70ml) was prepared in the standard manner[9] from minced hogliver (700g) without any further purification. The authors are indebt to Mrs.Wattiaux (Medical Faculty,Namur) for her help for the preparation of the microsomal solution.
9. S.H.Hogebomm,Methods in Enzymology,vol.1,p.16,Acad.Press,N.Y.(1955),S.P.Colowich,N.O.Kaplan,eds.
10. The structure of other products is under investigation
11. Based on the conversion of one optical isomer
12. Radiochromatographies were performed on an automatic Thin Layer Scanner II Berthold, LB 2723
13. We have performed a blank reaction omitting addition of labelled oxidosqualene 4b under conditions similar to those just described. The corresponding Fraction B' analysed by |GC|^2 is a complex mixture of products but the chromatogram is flat : a) between 14 and 19 min., b) in this region.
14. We sincerely acknowledge Prof. Verzele and Prof. Van de Walle, Rijksuniversiteit of Gent (Belgium) for the facilities offered for |GC|^2 and |GC|^{2}MS analysis and helpful discussions.
15. The minor peak (∿7%) is located at similar Rt as the one of *20R* norlanosteryl acetate 8a. The |GC|^{2}MS analysis seems to agree with this proposal. This is probably due to the presence of about 5% of the 18E isomer 4a in our sample[7] of 4b
16. M. Verzele and P. Sandra, J. Chromatogr., 158, 111 (1978)
17. This mother liquor yielded on evaporation solid material, which was recrystallized once using the same solvent system. (a) leads to crystals : (1750); mother liquor (2.300); (b) leads to crystals (1350); mother liquors (1850).
18. Under identical experimental conditions as described for *20S* analogues : (specific activity in dpm/mg) : 8a starting : (1760); crystals respectively : (680),(320),(200); mother liquors : (3000),(1100),(500). 9a[19] starting : (740); crystals respectively : (340),(150),(80); mother liquors : (2400),(1400),(650).
19. 8,9-oxido-norlanosteryl acetates decompose to some extent during cocrystallization experiments
20. We are investigating the behaviour toward squalene sterol cyclase, of squalene analogues with complete carbon framework but possessing : a) double bonds with Z configuration or b) internal nucleophilic moieties able to trapp intermediates.
21. The norsqualene 4a bearing the natural all trans stereochemistry, stereospecifically synthesized and subjected to the reaction with squalene sterol cyclase leads to the stereospecific formation of the norlanosterol 7a with the natural *20R* stereochemistry.
22. Ionic intermediates have also been formulated as bridged (non classical) carbonium ions. These are described by the authors (ref. 2c, p. 354) "merely as a graphic symbol for the stereospecificity of this cyclisation".
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