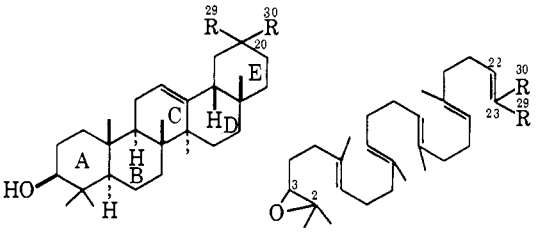
Direct Biosynthesis of the 29,30-Bisnoramyrin System from 29,30-Bisnor-2,3-oxidosqualene in Pea Seedlings

Sir:

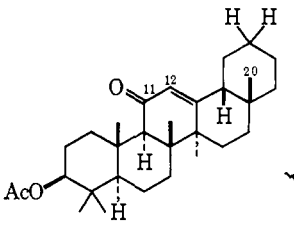
Evidence has been presented previously that β-amyrin (**1**) is formed biosynthetically in pea seedlings (*Pisum sativum*) from 2,3-oxidosqualene (**2**) in a non-oxidative process.[1] These findings, which are analogous to earlier results on the biosynthesis of lanosterol from 2,3-oxidosqualene[2,3] in liver microsomes, pave the way for detailed studies of the intricate series of enzymic cyclization and rearrangement steps which lead to the β-amyrin (oleane), α-amyrin (ursane), and other classes of pentacyclic triterpenes, especially since solubilization of the enzyme system was readily achieved for the β-amyrin case. The study reported here was initiated in order to ascertain whether the use of a 2,3-oxidosqualene analog lacking the two methyl groups which correspond to carbons 29 and 30 of α- and β-amyrin would cause a change (or interruption) in the enzymic cyclization and/or rearrangement sequences which normally produce these substances. The omission of these particular carbons from an oxidosqualene-like substrate constitutes a major structural perturbation in the region of the most complex and interesting processes in amyrin biosynthesis, those affecting the development of the D and E rings with their pendant groups.

dl-29,30-Bisnor-2,3-oxidosqualene (**3**), labeled with ^{14}C at the terminal methylene position (C_{23}), was synthesized from 2,3:22,23-dioxidosqualene[4,5] by a sequence involving (1) selective monohydration to the corresponding 2,3-oxido-22,23-diol,[6] (2) glycol cleavage with sodium periodate to the trisnoroxido aldehyde[6] and Wittig condensation with methylenetriphenylphosphorane labeled at the methylene group with ^{14}C. Unlabeled **3** was also prepared for characterization: mol wt (mass spectrometric) 398; nmr spectrum as expected for the assigned structure; R_f (thin layer chromatographic, tlc) on silica gel (CH_2Cl_2) 0.6, essentially the same as for 2,3-oxidosqualene (**2**). *Anal.* Found: C, 84.09; H, 11.63. Anaerobic incubation of the ^{14}C-labeled substrate **3**, which had a specific activity of 3.6×10^6 dpm/μmole, with a particle-free solution of 2,3-oxidosqualene–amyrin cyclase from pea seedlings[1,7] in pH 7.4 phosphate buffer (0.1 *M*) followed by ether extraction and tlc separation on silica gel (CH_2Cl_2) gave a product containing 15% of the radioactivity initially in the starting substrate in a zone centered at R_f 0.25 along with unchanged **3** at R_f 0.60. Acetylation (acetic anhydride–pyridine) of the new product yielded an acetate of R_f 0.60 (silica gel layer–CH_2Cl_2), very close to that of β-amyrin acetate. This acetate was shown to be chromatographically identical with an authentic sample of the acetate ester of 29,30-bisnoramyrin (**4**). The acetate of **4** was synthesized unambiguously from 29,30-bisnorolean-12-en-20-on-3β-yl acetate[8] in 58% yield by conversion to the 20-*p*-toluenesulfonylhydrazone followed by reduction with sodium borohydride in dioxane at reflux;[9] for the acetate of **4**: mp 218–220°; mol wt (mass spectrometric) 440; infrared max 5.78, 8.1 μm



1, R = CH_3
4, R = H

2, R = CH_3
3, R = H



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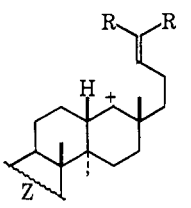
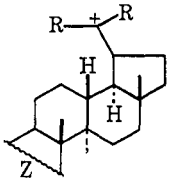
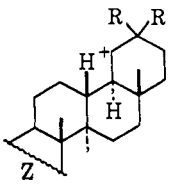


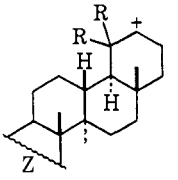
6, R = H



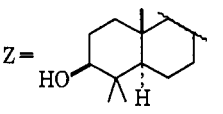
7, R = H



8, R = H



9, R = H



(acetate). *Anal.* Found (acetate of **4**): C, 80.66; H, 11.13. Admixture of authentic acetate of **4** with the acetate of the enzymic product from **3** and recrystallization four times from hot ethanol–water (3:1) yielded crystalline material showing 266, 148, 146, and 151 counts/(min mg), the constancy over the last three recrystallizations indicating identity of the radioactive acetate with authentic **4**-acetate. Further confirmation of this conclusion was obtained as follows. Oxidation of the acetate ester of **4** with chromic acid in acetic acid at reflux afforded the 11-oxo derivative **5**:[10] mp 218°; λ_{max} (95% ethanol) 249 nm (ε 11,000); mol wt (mass spectrometric) 454.3442. Similar oxidation of the acetate of the radioactive enzymic product from **4** yielded a product with R_f identical with that of **5** (0.24; silica gel–CH_2Cl_2) which was admixed with synthetic **5** and recrystallized four times from aqueous ethanol to

(1) E. J. Corey and P. R. Ortiz de Montellano, *J. Am. Chem. Soc.*, **89**, 3362 (1967).
(2) (a) E. J. Corey, W. E. Russey, and P. R. Ortiz de Montellano, *ibid.*, **88**, 4750 (1966); (b) E. J. Corey and W. E. Russey, *ibid.*, **88**, 4751 (1966).
(3) E. E. van Tamelen, J. D. Willet, R. B. Clayton, and K. E. Lord, *ibid.*, **88**, 4752 (1966).
(4) E. E. van Tamelen and T. J. Curphey, *Tetrahedron Letters*, 121 (1962).
(5) E. J. Corey and S. Gross, *J. Am. Chem. Soc.*, **89**, 4561 (1967).
(6) The experimental procedures were developed by Mr. B. E. Ganem in these laboratories and will be described in a forthcoming publication.
(7) The cyclase could also be extracted from sprouted pea homogenate without the aid of sodium desoxycholate (*cf.* ref 1). In a typical incubation 7 nmol of labeled substrate and enzyme solution from the homogenate of *ca.* 50 germinated peas were utilized.
(8) E. J. Corey and E. W. Cantrall, *J. Am. Chem. Soc.*, **81**, 1745 (1959)
(9) L. Caglioti and P. Grasselli, *Chem. Ind.* (London), 153 (1964).
(10) E. J. Corey and J. J. Ursprung, *J. Am. Chem. Soc.*, **78**, 183 (1956).

give successively crystals of radioactivity 268, 226, 225, and 226 counts/(min mg).

The enzymic formation of 29,30-bisnoramyrin (**4**) from 29,30-bisnor-2,3-oxidosqualene (**3**) is remarkable for a number of reasons. The cyclization which generates ring E, presumably involving **6** → **7** → **8**, or **6** → **9** (or their covalently coordinated cation equivalents),[11] proceeds regardless of whether R is methyl or hydrogen.[12] If **7** is an intermediate (as proposed for the biosynthesis of β-amyrin[11b]) a *primary* cation or its equivalent must be involved. On the other hand, if the E ring is formed *via* process **6** → **9** (as proposed for the biosynthesis of α-amyrin[11b]), then further conversion of **9** to **4** must involve a change in at least one of the groups (H instead of CH_3) undergoing migration to generate the amyrin system enzymically.[13] It should be possible to distinguish between these interesting alternatives experimentally, and such tests are planned. The study of substrate **3** with cyclase that produces *exclusively* α- or β-amyrin is also of interest, as is the extension to other enzymic systems such as lupeol or taraxerol cyclases.

The capacity of enzymes which normally produce pentacylic triterpenes from 2,3-oxidosqualene to handle other substrates raises the question as to the limits of enzyme specificity with regard to changes in substrate structure as well as other intriguing questions and opportunities for new research.[14]

(11) (a) L. Ruzicka, A. Eschenmoser, and H. Heusser, *Experientia*, **9**, 357 (1953); (b) A. Eschenmoser, L. Ruzicka, O. Jeger, and D. Arigoni, *Helv. Chim. Acta*, **38**, 1890 (1955).

(12) Since both α- and β-amyrins are formed in *Pisum sativum* (the latter in larger amount), both α- and β-cyclases may be present in the soluble enzyme preparation used in the above work. Clearly either might effect the conversion of **3** to **4**, and intermediates of type **8** and **9** are equally plausible at this time.

(13) As has been pointed out previously,[10,11b] the appropriate sequence of 1,2-*cis* migrations from cations **7** or **9** leads to the correct stereochemistry of α- and β-amyrins.

(14) This work was supported by the National Science Foundation and the National Institutes of Health.

(15) Radcliffe Institute Scholar, 1966–1968.

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Structure of the $C_2H_5O^+$ Ion in the Mass Spectra of 2-Alkanols

Sir:

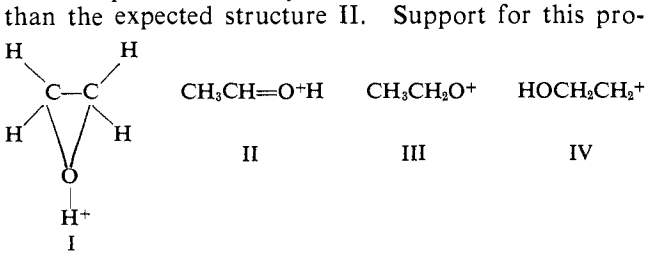
From deuterium labeling and energetics studies of fragmentation reaction 1 in 2-alkanols, Van Raalte and Harrison[1] suggested that the $C_2H_5O^+$ ions fragmenting

$$C_2H_5O^+ \longrightarrow H_3O^+ + C_2H_2 \qquad (1)$$

had the protonated ethylene oxide structure I rather than the expected structure II. Support for this proposal has been advanced by Shannon and McLafferty[2] who studied the metastable peaks observed at *m/e* 8.02 and 18.7 for reactions 1 and 2, respectively. They

$$C_2H_5O^+ \longrightarrow HCO^+ + CH_4 \qquad (2)$$

found the ratio of intensities *m/e* 8.02:*m/e* 18.7 to be constant for $C_2H_5O^+$ ions derived from 2-alkanols ($CH_3CH(OH)Y$), ethoxy derivatives (CH_3CH_2OY), and β-substituted ethanols ($HOCH_2CH_2Y$). On a structural basis one might expect the $C_2H_5O^+$ ions from the three classes to have structures II, III, and IV, respectively. They also observed that the metastable peak at *m/e* 18.7 was "flat-topped" in all cases, with the energy release identical within experimental error. To explain their results they proposed that the $C_2H_5O^+$ ions derived from the three classes of compounds had undergone rearrangement to a common intermediate, probably I, prior to fragmentation.



I

$CH_3CH{=}O^+H$ $CH_3CH_2O^+$ $HOCH_2CH_2^+$

II III IV

In an attempt to obtain definitive evidence concerning the structure of the $C_2H_5O^+$ ion derived from 2-alkanols we have examined the mass spectrum of 2-propanol-2-^{13}C.[3] Mass spectra were obtained at high resolution (~15,000) using an AEI MS-902 mass spectrometer. Table I records the ion current ratios for the ^{13}C-labeled ionic species and the corresponding unlabeled ion for the molecule ion, the $C_2H_5O^+$ ion, and the HCO^+ ion at 70-eV ionizing electron energy.

Table I. Relative Intensities at 70 eV

Ion ratio	Ratio of intensities
$^{13}CC_2H_5O^+ : C_3H_5O^+$	0.186
$^{13}CCH_5O^+ : C_2H_5O^+$	0.175
$^{13}CHO^+ : CHO^+$	0.118

The lower ^{13}C content of the $C_2H_5O^+$ is in good agreement with that expected for loss of a CH_3 group with natural ^{13}C abundance. The ^{13}C content of the CHO^+ ion was studied as a function of electron energy and found to decrease to $^{13}CHO^+ : CHO^+ = 0.108$ in the 30–20-eV region and then remain constant to 12 eV (all energies nominal values), the lowest energy at which significant intensities could be observed.[4]

The 70-eV data lead to 74% ^{13}C retention in the CHO^+ ion derived from $^{13}CCH_5O^+$, while the low-energy data lead to 68% ^{13}C retention. Neither value agrees with that predicted for the fragmentation of structure I or structure II alone. Fragmentation from the symmetrical structure I should lead to 50% ^{13}C retention, while fragmentation from structure II should lead to 100% ^{13}C retention.

In contrast to the ratio $^{13}CHO^+ : CHO^+ = 2.84$ at 70 eV for fragmentation reactions 3 and 4, the "metastable" intensities observed for fragmentations occurring in the

(1) D. Van Raalte and A. G. Harrison, *Can. J. Chem.*, **41**, 3118 (1963).

(2) T. W. Shannon and F. W. McLafferty, *J. Am. Chem. Soc.*, **88**, 5021 (1966).

(3) Prepared by the $LiAlH_4$ reduction of acetone-2-^{13}C with final purification by gas chromatography.

(4) The fragmentation pathway $(CH_3)_2CHOH^+ \rightarrow (CH_3)_2C{=}OH^+ + H \rightarrow C_2H_4 + CH_2OH^- \rightarrow CHO^+ + H_2$ can also lead to formation of CHO^+. The CH_2OH^+ intensity in 2-propanol is quite low and by comparison with the high-resolution spectrum of *t*-butyl alcohol, which fragments by a similar mechanism, we estimate that at 70 eV only 7% of the CHO^+ ion would originate by the above mechanism. Since the ^{13}C retention in CH_2OH^+ ($^{13}CH_2OH^+ : CH_2OH^+ = 0.107$) is similar to that observed for CHO^+, this contribution does not alter the interpretation of the results. Further, the onset potential for formation of CHO^+ by the above route should be more than 2 eV higher than formation of CHO^+ through reaction 2 and therefore should make a negligible contribution at low energies.