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CHEMICAL GENETICS OF A SUB-GLAUCOUS MUTANT OF *BRASSICA OLERACEA*

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Abstract—A complete chemical analysis of the wax from a sub-glaucous mutant of *Brassica oleracea* has been made. The major difference in chemistry is that *anteiso*-members are included into the long chain fatty acids, aldehydes, hydrocarbons and probably secondary alcohols. *Anteiso*-members have not previously been reported in these components in a *Brassica* wax. It is concluded that the sub-glaucous appearance of this mutant is due to the disruption of the crystalline structure on the leaf surface by the inclusion of an *anteiso*-component into the hydrocarbon fraction. The chemistry of this mutant has some bearing on the mode of biosynthesis of the C_{29} compounds and other aspects of wax biosynthesis.

INTRODUCTION

EARLY chemical analyses of plant waxes supported the head-to-head condensation mechanism for the synthesis of long-chain paraffins in many plant species and the C_{29} components of *Brassica oleracea* wax. Thus, in 1929 Clenshaw and Smedley-Maclean[1] proposed this mechanism to account for the preponderance of C_{27}, C_{31} and C_{35} paraffins that had then been reported. At the same time Channon and Chibnall[2] presented a similar hypothesis to account for their observation of nonacosane and 15-nonacosanone in cabbage leaves. They did, however, express reservations in this paper due to the lack of evidence for the existence of pentadecanoic acid in natural products. In a review of their work they[3] abandoned the condensation hypothesis in favour of a scheme that envisaged that the wax components in plants and insects were derived from very long chain (C_{24}–C_{38}) unsaturated fatty acids. Macey and Barber,[4] however, recently found pentadecanoic acid in the wax from glaucous *Brassica* and its marked reduction, along with the C_{29} compounds, in non-glaucous mutants.

Kolattukudy[5–8] has performed a series of experiments, utilizing radioactive tracers with brocolli leaves that strongly suggest a chain elongation decarboxylation mechanism. In his hypothesis[5,6] he proposed that a C_{16} acid was elongated to C_{30} and subsequently decarboxylated to give the C_{29} paraffin. The C_{29} ketones and secondary alcohols were believed to be synthesized by insertion of a functional group into the chain, when it is longer than

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† Professor H. N. Barber, F.R.S., died on the 16th April, 1971, before the completion of the manuscript. We dedicate this paper to the memory of a great natural philosopher.

[1] E. CLENSHAW and I. SMEDLEY-MACLEAN, *Biochem. J.* **23**, 107 (1929).
[2] H. J. CHANNON and A. C. CHIBNALL, *Biochem. J.* **23**, 168 (1929).
[3] A. C. CHIBNALL and S. H. PIPER, *Biochem. J.* **28**, 2209 (1934).
[4] M. J. K. MACEY and H. N. BARBER, *Nature, Lond.* **222**, 789 (1969).
[5] P. E. KOLATTUKUDY, *Biochem.* **5**, 2265 (1966).
[6] P. E. KOLATTUKUDY, *Phytochem.* **6**, 967 (1967).
[7] P. E. KOLATTUKUDY, *Plant Physiol.* **43**, 1466 (1968).
[8] P. E. KOLATTUKUDY, R. H. JAEGER and R. ROBINSON, *Nature, Lond.* **219**, 1038 (1968).

C_{18}, at a position corresponding to C_{15} in the final product. The final decarboxylation is presumably the same system as that utilized in the C_{29} paraffin synthesis.

This hypothesis can explain most of the results obtained by Macey and Barber in their analyses of waxes from glaucous *Brassica* and various non-glaucous mutants.[9] Thus in the mutant gl_4 they observed an excess of C_{30} acid and C_{30} aldehyde together with a loss of C_{29} compounds. This can be explained by assuming a mutation in the decarboxylation of C_{30} acid and its diversion into C_{30} aldehyde. In the mutant gl_2, which also lacks C_{29} compounds, they observed a loss of C_{30} acid and C_{30} aldehyde with an increase in C_{26} and C_{28} acids and aldehydes. This suggests a block in the elongation system. Robinson[10] has suggested that the C_{15} acid is a breakdown product of the ketone but this idea has not as yet been tested.

Hallam[11] has studied the surface wax of several species of *Eucalyptus* by electron microscopy. He concluded that the morphology of the crystallites is dependent upon wax chemistry since recrystallization, from cold acetone, of an extracted wax gave crystallites of a similar appearance to those found on the leaf surface. A new subglaucous mutant of kale, known as gl_6, has been acquired recently.[12] Its appearance is quite different to the non-glaucous mutants in that the leaf has an iridescent quality. The probable dependence of crystallite morphology on wax chemistry led us to believe that the wax chemistry of gl_6 might be unusual.

RESULTS

General

Table 1 shows the approximate percentage composition of gl_6 wax. These figures were obtained by weighing the components isolated using column chromatography on thin-layer silica gel as previously described.[13] An analysis of wax from glaucous plants is

TABLE 1. PERCENTAGE COMPOSITION OF WAXES FROM NORMAL AND gl_6 LEAVES

Components	Normal[9] (%)	gl_6[13] (%)
Hydrocarbons	33	40
Esters	19	7
Aldehydes		4
Ketone	20	20
Secondary alcohol	8	11
Unknown	trace	2
Primary alcohol	12	7
Free acid	8	8

[9] M. J. K. MACEY and H. N. BARBER, *Phytochem.* **9**, 13 (1970).
[10] R. ROBINSON, unpublished communication.
[11] N. D. HALLAM, *Planta* **93**, 257 (1970).
[12] K. F. THOMPSON, Plant Breeding Institute, Cambridge.
[13] A. G. NETTING, *J. Chromatog.* **53**, 507 (1970).

included for comparison. These figures are taken from Macey and Barber[9] who isolated the components by a combination of column chromatography on Florosil and preparative TLC. The similarity of gl_6 wax to the wax from glaucous plants is striking since in all other *Brassica* mutants that have been examined, there is a marked drop in C_{29} components.[9]

Free Acids and Aldehydes

The free acids were examined as their methyl esters by GLC. It was immediately apparent that there was a series of non-normal acids present as well as the usual normal acids. These acids were shown to be *anteiso*-acids in the chain length range *anteiso*-C_{25} to *anteiso*-C_{31}. Such a series of free acids has not been previously observed in a *Brassica* wax. The major components of the gl_6 free acids were: *n*-C_{26} (5·1%), *a*-C_{27} (4·2%), *n*-C_{28} (27·4%), *a*-C_{29} (29·8%), *n*-C_{30} (22·7%), *a*-C_{31} (2·5%).* Branched chain acids could not be detected in the C_{14}–C_{18} range.

After oxidation and methylation of the aldehydes, GLC suggested the presence of normal and *anteiso*-components. The tentative composition of the aldehyde fraction from gl_6 wax was: *n*-C_{26} (0·8%), *a*-C_{27} (4·9%), *n*-C_{28} (23·1%), *a*-C_{29} (19·2%), *n*-C_{30} (43·8%), *a*-C_{31} (4·7%). Small amounts of normal odd numbered components were just distinguishable from the *anteiso*-series.

Macey and Barber[9] found a close correspondence in composition between the aldehydes and free acids from the same wax from both glaucous and non-glaucous leaves. The present results extend this observation to *anteiso*-components. This again suggests a close relationship between free acids and aldehydes.

Esters and Primary Alcohols

The esters were hydrolysed and the acids obtained were examined as their methyl esters on GLC. The chain length range was C_{14}–C_{20} with traces up to C_{25}: normal, *iso*- and *anteiso*-members were present. The major components were *n*-C_{16} (8·4%), *a*-C_{17} (26·0%), *i*-C_{18} (8·8%), *n*-C_{18} (7·1%) and *a*-C_{19} (39·0%). The ester acids of glaucous cauliflower have been shown[14] to cover a similar chain length range with *n*-C_{16} (6·8%), *a*-C_{17} (16·6%), *i*-C_{18} (2·5%), *n*-C_{18} (12·4%), *a*-C_{19} (47·0%) and *n*-C_{20} (13·4%) as the major components.

The ester alcohols were examined directly by GLC and these also contained normal, *iso*- and *anteiso*-components, but were of a longer chain length than the ester acids. The major components were *a*-C_{23} (3·0%), *n*-C_{24} (4·7%), *a*-C_{25} (1·7%), *i*-C_{26} (6·6%), *n*-C_{26} (24·5%), *a*-C_{27} (25·7%), *i*-C_{28} (2·3%), *n*-C_{28} (10·1%), *a*-C_{29} (17·5%). This is similar to glaucous kale,[14] and also to the free primary alcohols of gl_6 wax. Here the major components were: *n*-C_{24} (6·2%), *a*-C_{25} (2·3%), *i*-C_{26} (2·4%), *n*-C_{26} (35·0%), *a*-C_{27} (12·5%), *i*-C_{28} (7·0%), *n*-C_{28} (12·9%), *a*-C_{29} (14·3%) and *n*-C_{30} (4·6%). The composition of the free primary alcohols from normal glaucous kale and normal glaucous cauliflower is again similar.[14]

Thus the mutation that produces the sub-glaucous appearance in gl_6 has little effect on the composition of the esters and primary alcohols of the wax. This is essentially similar to the results obtained with other mutations.

Unknown

The technique of column chromatography on thin-layer silica gel[13] allowed the isolation

* Abbreviations: *n*—normal; *a*—*anteiso*-; *i*—*iso*-.

[14] M. J. K. Macey, Ph.D. Thesis, University of N.S.W. (1967).

of a new component from gl_6 wax. GLC showed that the four major peaks present formed a homologous series, and by comparison with the C_{29} ketone from gl_6 wax, these were possibly C_{24}, C_{26}, C_{28} and C_{30}. An IR spectrum showed twin carbonyl peaks and was compatible with a keto-aldehyde. However a positive identification was not possible since a sufficient amount of the pure component was not available.

Hydrocarbons

The hydrocarbons were examined by GLC and the following components found: C_{27} (0·1%), C_{28} (0·8%), C_{29} (85·9%), C_{30} (7·2%), C_{31} (6·0%). It was apparent by the comparison with standards that all the components were normal except C_{30}. The fact that *anteiso*-components were found in the acids and aldehydes suggested that this C_{30} component may be *anteiso*-C_{30}. This was checked using Linde Molecular Sieve 5A.[15] After conditioning with branched chain standards[16] the molecular sieve absorbed all components except C_{30}, showing it to be branched chain. Assignment as *anteiso*-C_{30} was checked by co-chromatography, both with and without molecular sieve, with tobacco wax hydrocarbons which are rich in *anteiso*-C_{30}.[17]

Ketones and Secondary Alcohols

The composition of these two fractions was determined as previously described.[18] The ketone contained 94% 15-nonacosanone and 6% 14-nonacosanone, while the secondary alcohol contained 62% 15-nonacosanol and 38% 14-nonacosanol. In GLC there was a small shoulder (approx. 1% of total mass) on the trailing edge of the nonacosanol peak. Its position relative to the nonacosanol peak suggested that it might be *anteiso*-C_{30}. There was no sign of a similar component in the ketone fraction.

DISCUSSION

It seems highly likely that the glaucousness of normal *Brassica* leaves is due to the C_{29} hydrocarbon since glaucous leaves contain 153 mg of nonacosane per kg fresh wt. (approx. 33% total wax) while non-glaucous leaves contain from 1·0 to 8·5 mg (5–10% total wax).[9] Weitkamp[19] has shown that the introduction of an *iso*- or *anteiso*-branch into a fatty acid methyl ester has a profound effect on crystal structure. It appears likely that the presence of small amounts of *anteiso*-C_{30} in the hydrocarbon fraction of gl_6 wax is responsible for a loss of crystalline structure and thereby for the iridescent appearance.

Kolattukudy[5] was unable to find long chain free acids in the surface wax but did find evidence for these components in the phospholipids.[6] We have consistently been able to extract long chain free acids with the surface wax using hot light petroleum, as have other authors using the same solvent[20] or cold ether.[21] We feel that very long acids probably exist in the phospholipid fraction of the internal lipid since previous study has shown that a microsomal system is specific for the elongation process and a large proportion of the elongation products were in phospholipid.[22] Nevertheless in leaves synthesizing wax from

[15] B. T. WHITHAM, *Nature, Lond.* **182,** 391 (1958).
[16] D. T. DOWNING, Z. H. KRANZ and K. E. MURRAY, *Austral. J. Chem.* **13,** 80 (1960).
[17] T. KANEDA, *Biochem.* **7,** 1194 (1968).
[18] A. G. NETTING and M. J. K. MACEY, *Phytochem.* **10,** 1917 (1971).
[19] A. W. WEITKAMP, *J. Am. Chem. Soc.* **67,** 447 (1945).
[20] D. H. S. HORN, Z. H. KRANZ and J. A. LAMBERTON, *Austral. J. Chem.* **17,** 464 (1964).
[21] S. JEAN PURDY and E. V. TRUTER, *Proc. Roy. Soc. Lond.* **B158,** 536 (1963).
[22] M. J. K. MACEY and P. K. STUMPF, *Plant Physiol.* **43,** 1637 (1968).

palmitate-1-^{14}C, long chain fatty acids were present in the free acid portion of the wax and could not be detected in the internal lipid.[23] Furthermore these acids include a labelled C_{30} component not previously mentioned by Kolattukudy, and it is this which is possibly very closely related to the C_{29} compounds and to the C_{30} aldehyde of the wax. It does not follow of course that the very long chain acids are direct precursors of the C_{29} compounds and of the aldehydes, but only that they are closely related to the elongation system that produces them.

It has been suggested[24] that the primary alcohols of *Brassica* waxes are synthesized by the reduction of the coenzyme A derivatives of long chain acids, the aldehydes being an intermediate. Since the primary alcohols of all *Brassica* waxes that we have examined are rich in branched chain members[14] and, except in gl_6 wax, the long chain free acids and aldehydes lack branched chain components,[9] we believe that the pool of which the long chain free acids are representative cannot be the sole source of the primary alcohols. However, we do believe that the aldehydes are probably formed from the same pool as the long chain free acids since the composition of these two components is very similar.

Purdy and Truter[25] have examined the free primary alcohols as well as the acids and primary alcohols derived from the saponification of the esters from a *Brassica* wax. They found that all the members of these components were straight chain in disagreement with our results. We feel that their TLC methods gave inadequate resolution to distinguish straight and branched chain members. However, they did find a close resemblance between the composition of the free and ester primary alcohols; a correlation with which we agree. It is possible, then, that both the free and ester alcohols are derived from the same pool.

Kolattukudy[26] has postulated two mechanisms for the biosynthesis of the wax esters. Firstly he has found evidence for a transacylation system that transfers fatty acids from phospholipids to primary alcohols. If this is the case one would expect the phospholipids to be rich in branched chain acids. There is no evidence that any branched chain acids are present in *Brassica* phospholipid though an exhaustive search has not yet been made. Secondly Kolattukudy[26] describes an enzyme system that requires palmityl-CoA for the esterification of steryl alcohol. If this system can be shown to esterify branched chain acids in the C_{14}–C_{20} chain length range with primary alcohols in the C_{23}–C_{29} range it is a likely candidate for the biosynthesis of the esters found in the surface wax.

Our chemical analyses lead us to believe that both the ester acids and the free and ester primary alcohols are formed by an elongation process utilizing acetate, since alternate members of the three series (*iso-*, *anteiso-* and normal) are always found. Evidence for such a system for the ester acids can be adduced from experiments utilizing isoleucine-U-^{14}C with chopped broccoli leaves.[27] Most of the counts were found to be in the esters and primary alcohols, components that we have found to be rich in branched chain members. After saponification of the wax the acids were found to be rich in labelled *anteiso*-C_{17} and *anteiso*-C_{19} members. These almost certainly came from the ester acids rather than, as Kolattukudy[27] implies, the free acids since the ester acids are rich in these two members.[14] Thus it is probable that these labelled branched acids were synthesized from the isoleucine by an acetate elongation mechanism and then incorporated into the esters.

[23] M. J. K. Macey, *Phytochem.* **9**, 757 (1970).
[24] P. E. Kolattukudy, *Ann. Rev. Plant Physiol.* **21**, 163 (1970).
[25] S. Jean Purdy and E. V. Truter, *Proc. Roy. Soc. Lond.* **B158**, 544 (1963).
[26] P. E. Kolattukudy, *Biochem.* **6**, 2705 (1967).
[27] P. E. Kolattukudy, *Plant Physiol.* **43**, 1423 (1968).

The inclusion of *anteiso*-members into a variety of wax components in gl_6 suggests that the mutation is responsible for a loss of specificity in an enzyme system at some early stage in wax biosynthesis. If the condensation hypothesis were true in its classical form the presence of *anteiso*-C_{30} hydrocarbon in the wax would require the presence of *anteiso*-C_{15} or *anteiso*-C_{16} free acids. No such acids could be found. Also significant amounts of *anteiso*-members in the ketones and secondary alcohols might be expected since these are postulated as intermediates in hydrocarbon synthesis. We could find no trace of *anteiso*-components in the ketone and only about 1% in the secondary alcohols, while 7% of the paraffin was *anteiso*-C_{30}. On the other hand, the elongation-decarboxylation hypothesis suggests that the long chain free acids are related to the precursors of the C_{29} components and that the ketones and hydrocarbons are end products of this biosynthetic pathway. The present results from gl_6 wax provide some support for this hypothesis in that the free acids are rich in *anteiso*-members and could therefore be related to the source of the *anteiso*-C_{30} hydrocarbon and presumed *anteiso*-C_{30} secondary alcohol.

We therefore feel that the chemical evidence, on the whole, is in agreement with the biochemical evidence in supporting the elongation–decarboxylation hypothesis. However, this hypothesis makes little reference to the esters, primary alcohols, aldehydes and free acids that are also present in the wax. In order to understand the role of these components in wax biosynthesis, the components of normal glaucous plants should be divided into two groups, not on the basis of chain length, but on the basis of whether or not branched chain members are present. The first group contains the ester acids, ester alcohols and free primary alcohols since these components always contain branched chain members. The second group contains the other wax components; that is, long chain fatty acids, aldehydes, hydrocarbons, secondary alcohols and ketones. This group typically does not possess branched chain components in *Brassica oleracea*, and thus its elongation system may be separate and distinct from that producing the first group. On the other hand the long chain acids in this group may normally be derived from intermediates of the elongation system that gives rise to the primary alcohols. In either case specificity has been lost for normal components in gl_6 and has allowed the inclusion of *anteiso*-components but the exclusion of *iso*-compounds in long chain fatty acids, aldehydes and paraffins.

EXPERIMENTAL

Wax extraction and fractionation. The wax was extracted as previously described[9] from mature leaves of gl_6 plants grown in the School of Botany gardens. It was fractionated by column chromatography on thin-layer silica gel.[13] In general GLC was carried out as previously described.[9]

Subsequent examination of isolated fractions. Free acids were methylated[28] and examined on GLC. They were characterized by co-chromatography with the methyl esters of the free acids from wool wax characterized by Downing *et al.*[16]

Aldehydes were examined directly by GLC to give the percentage composition. A sample of the aldehydes was oxidized by the method used by Purdy and Truter[29] for oxidizing secondary alcohols. The ethereal solution of fatty acids obtained was evaporated and applied to a Florisil column and eluted with EtOAc to remove impurities. The fatty acids were recovered by eluting the column with 4% HOAc in Et_2O.[30] These were then methylated[28] and characterized by co-chromatography with the methyl esters of the wool wax acids.

Primary alcohols were examined directly by GLC and characterized by comparison with the reduced mixture of wool wax acids described by Downing *et al.*[16]

Esters. After saponification in 5% KOH in 90% MeOH the solution was acidified and extracted with Et_2O. This prevented the formation of the emulsion that is usually obtained if the alkaline solution is

[28] L. D. METCALFE and A. A. SCHMITZ, *Analyt. Chem.* **33**, 363 (1961).
[29] S. J. PURDY and E. V. TRUTER, *Proc. Roy. Soc. Lond.* **B158**, 553 (1963).
[30] K. K. CARROLL, *J. Lipid Res.* **2**, 135 (1961).

extracted. The alcohols were then recovered by eluting them from a Florisil column with benzene.[9] Subsequently the acids were recovered by elution with 4% HOAc in Et_2O.[30] The ester acids were methylated and characterized as described above for the free acids, and the ester alcohols were characterized directly on GLC in the same way as the free alcohols.

Unknown. The IR spectrum of a melted film of this component was studied. The correlations used were collated from Bellamy[31] and Nakanishi.[32] It was also examined in GLC and the retention times of its components compared with the C_{29} ketone from gl_6 wax.

Hydrocarbons were analysed by GLC, and tentative assignments made by co-chromatography with the hydrocarbons from glaucous kale and tobacco wax. The assignments were further checked using Linde Molecular Sieve 5A. The procedure was similar to that described by Downing *et al.*[16] A stainless steel column 1/8 ×·8 in. was packed with Linde Molecular Sieve Type 5A and attached to the outlet of the gas chromatographic column. It was then conditioned to reject branched chain hydrocarbons by the repeated injections, as melted samples, of branched chain hydrocarbons. Injection of test samples showed that the molecular sieve completely retained straight chain hydrocarbons. The branched chain hydrocarbons used to condition the Molecular Sieve were synthesized from the corresponding alcohols (i-C_{20}, a-C_{21}, a-C_{25}, i-C_{26} and a-C_{27}) kindly supplied by Dr. K. E. Murray.* The conversion of alcohols to hydrocarbons was achieved by the method described by Kranz *et al.*[33] except that the iodides obtained as intermediates were purified on Florisil instead of alumina. The reduction of the iodides to the hydrocarbons was carried out in tetrahydrofuran rather than Et_2O, since the yields when using Et_2O as solvent were low. The purity of the hydrocarbons and the completeness of the reduction was tested by TLC on silica gel G with hexane as developing solvent.

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[31] L. J. BELLAMY, *The Infra Red Spectra of Complex Molecules* (2nd Edition) Methuen, London (1958).
[32] K. NAKANISHI, *Infra Red Absorption Spectroscopy—Practical.* Holden-Day, San Francisco; Nankodo, Tokyo (1962).
[33] Z. H. KRANZ, J. A. LAMBERTON, K. E. MURRAY and A. H. REDCLIFFE, *Aust. J. Chem.* **13**, 498 (1960).

Key Word Index—Brassica oleracea; Cruciferae; wax; genetics; *anteiso*-lipids.