Biosynthesis of the Furanoacetylene Phytoalexin Wyerone in *Vicia faba*

Nidhal A. Al-Douri and Paul M. Dewick

Department of Pharmacy, University of Nottingham, Nottingham, NG7 2RD, UK.

Z. Naturforsch. **41c**, 34–38 (1986); received June 24, 1985

Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

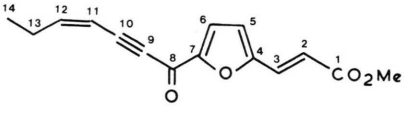
Biosynthesis, Phytoalexin, Furanoacetylene, Wyerone, *Vicia faba*

Feeding experiments using ^{13}C-labelled sodium acetate precursors in $CuCl_2$-treated broad bean *(Vicia faba)* cotyledons have demonstrated that the furanoacetylene phytoalexin wyerone is biosynthetically derived from seven intact acetate units. A further experiment using sodium [$^2H_3$]acetate indicated the head of the chain, and showed the chain is analogous to that of a fatty acid precursor, any chain shortening process from postulated C_{18} precursors occurring from the carboxyl end. Incorporations of oleate and linoleate were, however, regarded as insufficient to prove the involvement of these compounds in the biosynthetic pathway.

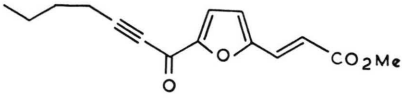
Introduction

In the vast majority of leguminous plants investigated, the phytoalexin response of this family results in the synthesis of isoflavonoid derivatives, especially pterocarpans and isoflavans [1]. The broad bean, *Vicia faba,* however, is unusual in that it synthesizes predominantly furanoacetylene phytoalexins when challenged by fungi or abiotic agents [1]. Although small amounts of the pterocarpan medicarpin have been identified in the phytoalexin fraction from this plant [2], the major component is the furanoacetylene ester wyerone (**1**) [3], accompanied by a number of structurally similar derivatives. These include the acid wyerone acid [4], the 11,12-epoxide wyerone epoxide [5], the 8-alcohol wyerol [3, 6], and 11,12-dihydro analogues, *e.g.* dihydrowyerone (**2**) [7]. Wyerone and wyerone epoxide have also been identified as phytoalexins from other species of *Vicia* and *Lens* [8, 9].

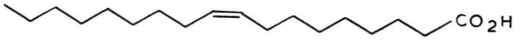
Wyerone is undoubtedly derived from acetate-malonate *via* the fatty acid pathway, and suggestions have been made [3] that the C_{18} acids oleate (**3**), linoleate (**4**) and crepenynate (**5**) may be involved in the biosynthesis, in common with many other acetylenic compounds [10, 11]. Initial feeding experiments using cotyledons of *Vicia faba* infected with *Botrytis cinerea* have confirmed the incorporation of acetate, malonate and oleate into wyerone [12]. In the later stages of the pathway, the sequence wyerol ⟶ wyerone ⟶ wyerone acid has been proposed [7] based on analysis of variations in levels of wyerone derivatives produced during the phytoalexin response. Considerably more experimental evidence is necessary to enable a logical biosynthetic pathway to be formulated for these wyerone derivatives. In particular, the C_{14} chain of wyerone is not readily related to the C_{18} chain of the proposed precursors oleate, linoleate and crepenynate without significant struc-



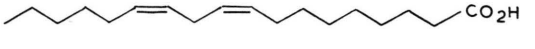
1 wyerone



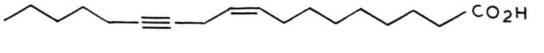
2 dihydrowyerone



3 oleic acid



4 linoleic acid



5 crepenynic acid

Reprint requests to Dr. Dewick.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/86/0100–0034 $ 01.30/0

tural modification. We report here the results of feeding experiments to clarify aspects of wyerone biosynthesis.

Results and Discussion

A biosynthetic pathway to wyerone *via* C_{18} fatty acids necessitates chain shortening at some stage and loss of four carbon atoms, perhaps by two β-oxidation sequences at the carboxyl end (Fig. 1a). However, the functionality of wyerone could be related better to that of crepenynate if chain shortening occurred at both ends, losing one carbon from the head of the chain and three from the carboxyl end (Fig. 1b). Any such speculation requires a knowledge of the location of biosynthetic acetate units in the wyerone chain, and these data can easily be obtained by the use of [^{13}C]acetate precursors and ^{13}C NMR analysis of the product.

Seeds of *Vicia faba* were surface sterilised, imbibed in water and the cotyledons were then removed and treated with aqueous $CuCl_2$ in the dark to stimulate phytoalexin biosynthesis [7]. Other abiotic agents, *e.g.* $HgCl_2$ and iodoacetate proved less satisfactory as inducers. After 7 days, the plant material was worked up, and wyerone isolated and purified by TLC. To ensure minimal degradation of the photolabile wyerone, all procedures were carried out in darkened conditions and samples were stored as ethanolic solutions in the dark at −20 °C. Wyerone concentration (approx. 150 μg/g tissue, wet wt.) was quantified by UV absorption [3] and its identity was confirmed by ^{1}H NMR (Table I) [3, 13]. This indicated the presence of small amounts (approx. 8%) of dihydrowyerone (**2**) (δ 0.92, t, J = 7 Hz, H−14) [13] which is not separated from wyerone by TLC methods. Whilst the compounds are readily resolved by HPLC [14], their separation was unnecessary for the experiments described here. Feeding experiments were carried out in the same manner, but supplying ^{13}C-labelled sodium acetate (1 g per 200 g *V. faba* cotyledons) five days after induction, then growing on for a further 48 h. Sodium [1-^{14}C]acetate was supplied together with the ^{13}C-labelled acetate to enable incorporation data to be calculated (Table II). The addition of these levels of sodium acetate usually enhanced wyerone synthesis (Table II) and thus production of unlabelled wyerone can also be significantly improved by adding unlabelled sodium acetate to the $CuCl_2$-treated cotyledons.

Assignment of the ^{13}C NMR spectrum of wyerone (Table III) was assisted by specific proton decoupling experiments, irradiating for H−2, H−3, H−5, H−6, H−11 and H−12. This allowed C−2, C−5, C−11 and C−12 to be assigned, but irradiation at 7.34 ppm (H−6) or 7.46 ppm (H−3) produced singlets for both ^{13}C signals at 121.2 and 130.0 ppm. Their as-

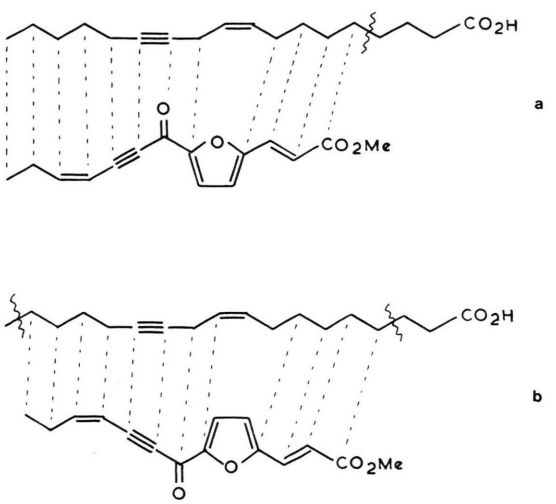


Fig. 1. Hypothetical biosynthetic relationships of crepenynic acid to wyerone.

Table I. ^{1}H NMR assignments for wyerone.

	δ [ppm]	Mult	J [Hz]
H−2	6.62	d	15.8
H−3	7.46	d	15.8
H−5	6.74	d	3.7
H−6	7.34	d	3.7
H−11	5.68	dt	10.8, 1.4
H−12	6.38	dt	10.8, 7.5
H−13	2.51	quint, d	7.6, 1.4
H−14	1.12	t	7.5

Table II. Incorporation data for wyerone from feeding experiments with labelled acetate.

	Wyerone μg/g fr.wt.	Dilution*	% Incorp*
sodium [1-^{13}C]acetate	113	43	0.016
sodium [2-^{13}C]acetate	204	29	0.044
sodium [1,2-$^{13}C_2$]acetate	279	54	0.034
sodium [$^2H_3$]acetate	220	28	0.049

* Calculated for sodium [1-^{14}C]acetate fed simultaneously.

Table III. ^{13}C NMR assignments for wyerone and spectral data from [^{13}C]acetate feeding experiments.

	δ [ppm]	Mult	Enriched [2-^{13}C]acetate	J $^{13}C-^{13}C$ [Hz] [$^{13}C_2$]acetate
C−1	166.5	s		77
C−2	120.7	d	+	76
C−3	130.0	d		69
C−4	154.8	s	+	69
C−5	115.7	d		52
C−6	121.2	d	+	52
C−7	153.6	s		79
C−8	164.5	s	+	79
C−9	90.5	s		
C−10	89.9	s	+	
C−11	106.4	d		71
C−12	153.8	d	+	71
C−13	24.7	t		34
C−14	13.1	q	+	34
OMe	51.9	q		

signments to C−6 and C−3 respectively was facilitated by the feeding experiment using [$^{13}C_2$]acetate, as were those for C−4/C−7 and C−1/C−8 which could not be assigned by chemical shifts alone. The acetylenic carbons C−9 and C−10 at 90.5 and 89.9 ppm respectively were allocated after the biosynthetic analysis and the results of the [2-^{13}C]acetate feeding experiment (Table III). Recovery levels of wyerone after the feeding of sodium [1-^{13}C]acetate proved too low to allow satisfactory assessment of enhanced signals, but the incorporation of sodium [2-^{13}C]acetate showed that seven carbons of the C_{14} chain were enriched (approx. 0.5%). Combined with the results from the [$^{13}C_2$]acetate experiment showing six pairs of coupled carbons (approx. 0.2% enrichment) and thus intact acetate units, this allowed the labelling pattern of Fig. 2 to be deduced. The acetylenic carbons can also be assumed to originate from a single acetate unit. Unfortunately, the similar δ values for C−9 and C−10, the very large coupling constant for acetylenic carbons (approx. 170 Hz [15]) and the low intensity signals for these quaternary carbons resulted in only one of each pair of satellites being visible. However, the data available are fully consistent and allow the carbon chain of wyerone to be related to that of a normal fatty acid. Any chain shortening process, if involved, presumably removes intact acetate units and would be consistent with a β-oxidation sequence.

Proof that C−14 of wyerone is in fact analogous to the methyl head of a fatty acid chain was obtained by a related feeding experiment using sodium [$^2H_3$]acetate. The ^2H NMR spectrum of wyerone from this feeding showed only one peak of significant intensity, at δ 1.10 ppm, corresponding to H−14. No labelling of other potential sites, H−12, H−6 or H−2 was observed. This result indicates that C−14/C−13 must be the starter acetyl CoA unit. This unit would experience little ^2H loss by exchange, but all extension units would undergo increased exchange of ^2H *via* malonyl CoA units or other exchange mechanisms [16−18]. Thus, the wyerone skeleton is derived from a fatty acid-type precursor and the C_{14} chain is analogous to the original fatty acid chain.

The possible involvement of oleic acid and linoleic acid was tested by feeding the [U-^{14}C]-labelled acids as their sodium salts (10 mg per 20 g cotyledons) and measuring incorporation data into wyerone. Sodium [1-^{14}C]acetate was used in a comparative experiment. The results (Table IV) show that these materials were not particularly effective precursors of wyerone, and dilution values were not significantly different from that of acetate. Ammonium oleate and methyl oleate were also rather poorly utilized. Whilst these results may be a consequence of poor absorption or transport of the material fed, they do raise the possibility that these compounds are not precursors

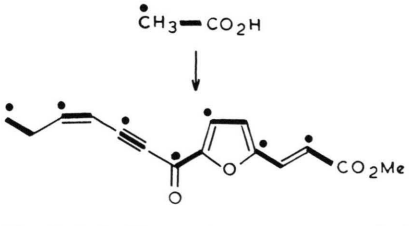


Fig. 2. Labelling pattern in wyerone derived from ^{13}C-acetate feeding experiments.

Table IV. Incorporation data for wyerone from feeding experiments with ^{14}C-labelled oleate and linoleate.

	Wyerone μg/g fr.wt.	Dilution	% Incorp
sodium [1-^{14}C]acetate	204	224	0.057
sodium [U-^{14}C]oleate	194	661	0.064
sodium [U-^{14}C]linoleate	178	126	0.31
ammonium [U-^{14}C]oleate	184	1160	0.034
methyl [U-^{14}C]oleate	184	467	0.086

of wyerone, except *via* initial degradation to acetyl CoA. Whilst wyerone formation is related to fatty acid metabolism, its biosynthesis from C_{18} acids and a route *via* crepenynic acid is not confirmed, and is structurally unlikely.

Wyerone does in fact bear a striking structural relationship to the aldehydes **7** from *Chrysanthemum silvaticum* [19] and **8** from *C. coronarium* [20]. However, these compounds have C_{13} and C_{12} chains respectively and biogenetically are believed to arise [11] from crepenynic acid and the ene-triyne acid **6** losing five carbon atoms from the carboxyl end together with the methyl end group in the case of **8** (Fig. 3). In this respect, wyerone must be considered biogenetically distinct, and further investigations are required.

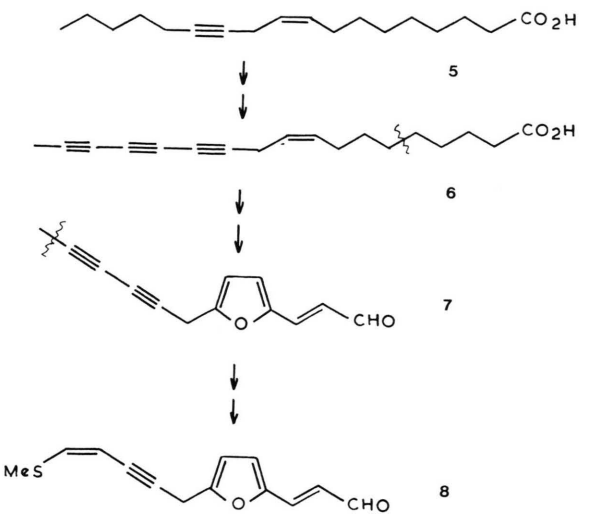


Fig. 3. Postulated biosynthetic origins of **7** and **8** from crepenynic acid **5** [11].

Experimental

NMR

NMR spectra were recorded using a Bruker WM250 spectrometer operating at 250 MHz (^{1}H), 62.89 MHz (^{13}C) or 38.4 MHz (^{2}H). Samples were dissolved in $CDCl_3$ (Me_2CO for ^{2}H spectra) and chemical shifts are relative to TMS.

Labelled compounds

Sodium [1-^{14}C]acetate (NEN, 2.4 mCi/mM), [U-^{14}C]oleic acid (NEN, 900 mCi/mM) and [U-^{14}C]linoleic acid (NEN, 900 mCi/mM) were purchased. Methyl [U-^{14}C]oleate was prepared from the acid using ethereal diazomethane. Sodium [1-^{13}C]acetate (90% ^{13}C), sodium [2-^{13}C]acetate (90% ^{13}C) and sodium [1,2-$^{13}C_2$]acetate (90% ^{13}C at each carbon) were purchased from Amersham, and sodium [$^2H_3$]acetate from Aldrich.

Plant material, feeding techniques and isolation of wyerone

Seeds of *Vicia faba* cv. Aquadulce were gently agitated in EtOH for 10 min, washed with H_2O and then immersed in dilute detergent (Quadralene) for 5 min. After washing, they were soaked in sodium hypochlorite (2%) for 5 min, washed again, and seeds visibly damaged during sterilisation were discarded. The remainder were left to imbide in distilled water for 24 h. After removal of the seed coat, cotyledons (200 g) were separated and placed flat side up on moist tissue paper in glass trays. Aqueous $CuCl_2$ (3 mM) was spotted onto the upper surfaces of the cotyledons using a syringe, adding sufficient to moisten the whole surface. The glass trays were then covered and kept at 25 °C in the dark, adding extra $CuCl_2$ as necessary to keep the surfaces moist. After 5 days, the inducer solution was carefully removed by syringe, and replaced with drops of the precursor solution (30 ml) containing sodium [^{13}C]acetate (1 g) and sodium [1-^{4}C]acetate (50 μCi). The cotyledons were left for a further 2 days, adding distilled water as appropriate. After this period, the cotyledons were homogenised in a blendor with CH_2Cl_2 (10 × 500 ml), filtering between extractions. The dichloromethane extract was concentrated, then chromatographed by TLC (silica gel GF_{254}; hexane-acetone, 2:1). Wyerone was detected as a major deep blue fluorescent zone under UV_{254}, and was eluted with acetone (Analar). The product was purified further by TLC using $CHCl_3$-petrol (2:1) and $CHCl_3$–MeOH (25:1). Wyerone had UV absorption λ_{max} (EtOH) 224, 290, 350 nm, lit [3] 226, 291, 351 (ε 27,000) nm, and was quantified using the latter absorption data. Yields were typically 50–55 mg/200 g fresh tissue. ^{1}H NMR data showed the presence of approximately 8% dihydrowyerone in this material.

Note: All manipulations were carried out in conditions of minimal lighting to avoid decomposition of wyerone. Samples were stored in EtOH solution in the dark at −20 °C.

Salts and esters of oleic and linoleic acids (1 μCi) were diluted with inactive material (10 mg) and fed to 20 g cotyledons as aqueous emulsions (1 ml) *via* the addition of Tween 20 (2 drops).

Acknowledgements

We thank Dr. J. L. Ingham (Reading) for reference material and the Iraqi Government for financial support (to N.A.A.).

[1] J. L. Ingham, in Phytoalexins (J. A. Bailey and J. W. Mansfield, eds.), p. 21, Blackie, Glasgow and London 1982.
[2] J. A. Hargreaves, J. W. Mansfield, and D. T. Coxon, Nature **262,** 318 (1976).
[3] C. H. Fawcett, D. M. Spencer, R. L. Wain, A. G. Fallis, E. R. H. Jones, M. Le Quan, C. B. Page, V. Thaller, D. C. Shubrook, and P. M. Whitham, J. Chem. Soc. (C), **1968,** 2455.
[4] R. M. Letcher, D. A. Widdowson, B. J. Deverall, and J. W. Mansfield, Phytochemistry **9,** 249 (1970).
[5] J. A. Hargreaves, J. W. Mansfield, D. T. Coxon, and K. R. Price, Phytochemistry **15,** 119 (1976).
[6] J. A. Hargreaves, J. W. Mansfield, and D. T. Coxon, Phytochemistry **15,** 651 (1976).
[7] J. W. Mansfield, A. E. A. Porter, and R. V. Smallman, Phytochemistry **19,** 1057 (1980).
[8] D. J. Robeson, Phytochemistry **17,** 807 (1978).
[9] D. J. Robeson and J. B. Harborne, Phytochemistry **19,** 2359 (1980).
[10] J. D. Bu'Lock and G. N. Smith, J. Chem. Soc. (C), **1967,** 332.
[11] F. Bohlmann, T. Burkhardt, and C. Zdero, Naturally Occurring Acetylenes, Academic Press, London and New York 1973.
[12] R. O. Cain and A. E. A. Porter, Phytochemistry **18,** 322 (1979).
[13] D. W. Knight and A. P. Nott, J. Chem. Soc. Perkin Trans. I, **1982,** 623.
[14] A. E. A. Porter, R. V. Smallman, and J. W. Mansfield, J. Chromatogr. **172,** 498 (1979).
[15] R. J. Abraham and P. Loftus, Proton and Carbon-13 NMR Spectroscopy, p. 54, Heyden, London 1978.
[16] B. Sedgwick and J. W. Cornforth, Eur. J. Biochem. **75,** 465 (1977).
[17] B. Sedgwick, J. W. Cornforth, S. J. French, R. T. Gray, E. Kelstrup, and P. Willadsen, Eur. J. Biochem. **75,** 481 (1977).
[18] A. G. McInnes, J. A. Walker, and J. L. C. Wright, Tetrahedron Lett., **1979,** 3245.
[19] F. Bohlmann, L. Fanghänel, K.-M. Kleine, H. D. Kramer, H. Mönch, and J. Schuber, Chem. Ber. **98,** 2596 (1965).
[20] F. Bohlmann, C. Arndt, H. Bornowski, K.-M. Kleine, and P. Herbst, Chem. Ber. **97,** 1179 (1964).