Phytochemistry, 1977, Vol. 16, pp. 1919–1923. Pergamon Press. Printed in England.

INTRACELLULAR DISTRIBUTION AND ORIGIN OF PENTACYCLIC TRITERPENES IN *CALENDULA OFFICINALIS* LEAVES

WIRGINIA JANISZOWSKA and ZOFIA KASPRZYK
Institute of Biochemistry, University of Warsaw, 02-089 Warszawa, al. Zwirki i Wigury 93, Poland

(*Received* 8 *March* 1977)

Key Word Index—*Calendula officinalis*; Compositae; triterpenes; intracellular distribution; labelling dynamics.

Abstract—In *Calendula officinalis* leaves the cyclization of squalene to β-amyrin and its further oxidation to oleanolic acid as well as the biosynthesis of all derivatives of oleanolic acid 3-glucoside and some derivatives of oleanolic acid 3-glucuronoside occur in the microsomal fraction. The final metabolites of oleanolic acid 3-glucoside series i.e. pentaglycosides, are translocated from this fraction, one to the cell wall and plasmalemma fraction and the other to the cytosol. The derivatives of oleanolic acid 3-glucoronoside are synthesized partially in other fractions and accumulate in the different membraneous structures of the cell.

INTRODUCTION

Oleanolic acid, formed in *Calendula officinalis* by oxidation of the 28-Me group of β-amyrin, does not occur in the leaves as the free acid but is found in the form of two glycoside derivatives, the 3-monoglucuronoside (glucuronosides) and the 3-monoglucoside (glucosides) [1, 2]. Three of the glucuronosides containing sugars exclusively at the 3-hydroxyl of oleanolic acid are the 3-glucuronoside (F), the 3'- (or 2'-)galactosyl-glucuronoside (D) and the 4'-glucosyl (3'-galactosyl)-glucuronoside (B) respectively. The remaining two glucuronosides (C and A) are glucoside esters of glycosides D and B respectively, containing an additional glucose residue bound to the C-17 carboxyl of the aglycone [3, 4]. The series of glucosides containing sugars exclusively at the 3-hydroxyl of oleanolic acid are the 3-glucoside (**1g**), the 3(4'-galactosyl)-glucoside (**2g**), 3(4'-digalactosyl)-glucoside (**3g**), 3(3'-diglucosyl, 4-digalactosyl)-glucoside (**6g**) and 3(3'-triglucosyl, 4'-galactosyl)-glucoside (**7g**).

Subsequent studies [5] have shown that pentaglycoside **6g** and, to a small extent, pentaglycoside **7g** are transported from *C. officinalis* leaves to the root, where they undergo gradual deglycosylation to oleanolic acid [6].

The aim of the present study was to examine the distribution and mevalonate [2-^{14}C]-labelling dynamics of pentacyclic triterpenes in cellular subfractions of *C. officinalis* leaves, to gain information on the site of biosynthesis of these compounds and on their translocation within the cell.

RESULTS AND DISCUSSION

In studies on the triterpene distribution in the cellular subfractions of *C. officinalis* leaves, each of the subfractions was obtained and characterized as previously described [7]. Glycosides were separated by chromatography and then hydrolyzed, their content of oleanolic acid was determined by gas chromatography of the methyl ester. Triterpene alcohols were quantitatively determined after TLC by acetylation with radioactive acetic anhydride, as previously reported [7]. As shown in ref. [7] radioactive squalene occurred mainly in the microsomal fraction (IV). In this fraction the radioactivity of squalene decreased from the beginning of the experiment, this pointing to rapid transformation of this compound both into sterols and β-amyrin.

The determinations have shown the presence of β-amyrin and erythrodiol in the microsomal (IV), mitochondrial –Golgi (III) and chloroplast (II) fractions, 78% of β-amyrin and 65% of erythrodiol being localized in fraction IV (Table 1).

While the investigated subfractions contained no free oleanolic acid, they contained its glycosides, whose distribution are also recorded in Table 1. The amount of oleanolic acid in the derivatives of oleanolic acid 3-glucoside was five times lower than that in the 3-glucuronoside derivatives. When listed in order of decreasing content, the glucosides assumed the following sequence: pentaglycoside **6g** (55%), monoglucoside **1g** (23%), second pentaglycoside **7g** (10%), triglycoside **3g** (7%), and diglycoside **2g** (5%). In fraction IV all the derivatives of oleanolic acid monoglucoside were present and constituted 42% of total cell glucosides. Fraction III contained glycosides **1g**, **3g** and **6g** (17% of total), fraction II contained glycoside **6g** (1%), fraction I contained only glycoside **7g** (7%) and fraction V had a large amount of glycoside **6g** and much smaller amount of glycoside **7g** (33%).

Different results were obtained for derivatives of oleanolic acid 3-glucuronoside. When listed in order of decreasing content, these compounds assumed the following sequence: diglycoside D (51%), triglycoside C (25%), second triglycoside B (15%), monoglucuronoside F (5%) and tetraglycoside A (4%). 61% of all glucuronosides were present in fraction III, 25% in fraction I, 9% in fraction II, 5% in fraction IV and less than 1% in fraction V. In fraction IV the presence of glycosides F, D and C was proved, while fraction III contained glycoside D in higher amount than glycoside C and also small amounts of glycosides B and A. In the fraction II and I all derivatives of oleanolic acid 3-glucuronoside, except

Table 1. The quantitative determinations of β-amyrin, erythrodiol, derivatives of oleanolic acid 3-glucuronoside and 3-glucoside in subcellular fraction of *C. officinalis* leaves*

Fraction protein mg*	I 2,3		II 0,4		III 0,9		IV 1,8		V 1,6		Sum 6,4	
Compound	μg	%	μg	%	μg	%	μg	%	μg	%	μg	%
β-Amyrin	0.00	0	0.12	5	0.42	17	1.96	78	0.00	0	2.5	100
Erythrodiol	0.00	0	0.04	20	0.03	15	0.13	65	0.00	0	0.2	100
Oleanolic acid in glycosides												
F	0.0	0	0.0	0	1.9	37	2.5	49	0.7	14	5.1	100
D	8.9	17	4.8	9	35.9	71	1.7	3	0.0	0	51.3	100
C	2.5	10	0.6	2	22.0	86	0.4	2	0.0	0	25.5	100
B	10.6	72	2.7	18	1.4	10	0.0	0	+	–	14.7	100
A	3.5	78	0.6	13	0.4	9	0.0	0	+	–	4.5	100
Total glucuronosides	25.5		8.7		61.6		4.6		0.7		101.1	
1g	0.0	0	0.0	0	0.7	15	4.1	85	+	–	4.8	100
2g	0.0	0	0.0	0	+	–	0.9	100	+	–	0.9	100
3g	0.0	0	0.0	0	1.1	73	0.4	27	0.0	0	1.5	100
6g	0.0	0	0.3	3	1.7	15	2.6	23	6.6	59	11.2	100
7g	1.5	75	0.0	0	0.0	0	0.5	25	+	–	2.0	100
Total glucosides	– 1.5		– 0.3		– 3.5		– 8.5		– 6.6		– 20.4	

* From 10 g of leaves.

monoglucuronoside, were present, whereas the soluble fraction contained glycoside F and and trace amounts of glycosides B and A.

In the investigations on both the sites of biosynthesis and translocation of triterpene alcohols and glycosides of oleanolic acid within the cell, isotope experiments were performed to study the dynamics of labelling of these compounds in the cellular subfractions. In Tables 2–7 the mean cpm values of the compounds studied in cellular fractions obtained from 10g of fresh leaves of *C. officinalis* are presented.

The total radioactivity incorporated into β-amyrin dropped from the beginning of the experiment and that of erythrodiol attained a maximum after 24 hr whereas the total radioactivity incorporated into oleanolic acid of all glycosides increased continually up to the end of the experiment. These results suggest that β-amyrin is oxidised via erythrodiol to oleanolic acid which, after glycosylation is accumulated in the plant. However distinct changes were observed in the labelling of its glucuronoside and glucoside derivatives. The radioactivity in the glucuronosides increased continually from 4 to 280 hr. The radioactivity of glucosides however passed a peak at 70 hr. At this time their radioactivity was twofold lower

Table 2. MVA-[2^{14}C] incorporation into pentacyclic triterpene alcohols and oleanolic acid isolated from the glycosides of *C. officinalis* leaves*

	Time							
	4 hr		24 hr		70 hr		280 hr	
Compound	Cpm	%	Cpm	%	Cpm	%	Cpm	%
β-Amyrin	59210		15900		11250		8830	
Erythrodiol	460		1480		730		540	
Oleanolic acid in glycosides								
1g	8430	43	5200	12	2790	3	1210	2
2g	6290	32	15950	38	6440	7	480	<1
3g	2630	13	12500	29	8160	9	4340	10
6g	1310	7	4440	10	42210	46	34910	64
7g	840	5	4610	11	31780	35	13320	24
Total	19500		42700		91380		54260	
F	98110	89	87790	74	5180	3	7240	3
D	11540	10	20490	17	124920	67	118530	51
C	360	<1	5820	5	21250	11	57200	25
B	290	<1	2350	2	26740	14	32620	14
A	220	<1	1980	2	9630	5	15470	7
Total	110520		118430		187720		231060	
Sum	130020		161130		279100		285320	

* From 10 g of leaves.

Table 3. MVA-[2^{14}C] incorporation into triterpenes in subfraction IV obtained from *C. officinalis* leaves*

Compound	Time			
	4 hr	24 hr	70 hr	280 hr
	cpm			
β-Amyrin	58800	14700	9260	6670
Erythrodiol	430	1400	630	340
Oleanolic acid in glycosides				
1g	7920	4180	2090	530
2g	6180	15540	6120	180
3g	2360	11460	5510	590
6g	1190	2910	32220	5530
7g	840	4010	27610	1930
F	82300	81700	1650	1520
D	10410	15200	97120	3920
C	60	90	1450	1700
B	0	0	0	0
A	0	0	0	0

* From 10 g of leaves.

Table 5. MVA-[2^{14}C] incorporation into triterpenes in subfraction II obtained from *C. officinalis* leaves*

Compound	Time			
	4 hr	24 hr	70 hr	280 hr
	cpm			
β-Amyrin	120	430	840	820
Erythrodiol	0	30	50	90
Oleanolic acid in glycosides				
1g	0	0	0	80
2g	0	0	0	0
3g	0	0	0	0
6g	0	210	1010	1860
7g	0	0	0	0
F	0	0	0	0
D	380	1120	5150	16440
C	0	1300	3020	5500
B	0	1100	11760	9410
A	0	210	720	2440

* From 10 g of leaves.

than that of glucuronosides, whereas it was fivefold lower at 4 and 280 hr. This fact indicates that glucosides are more metabolically active compounds than glucuronosides.

Changes in the radioactivity of individual glycosides are in agreement with the earlier proposed schemes of their transformations [4].

In fraction IV (Table 3) the time-course of labelling of β-amyrin and erythrodiol reflected the changes of total radioactivity of these compounds and indicate that in the microsomal fraction β-amyrin is formed as a result of squalene cyclization and subsequently undergoes oxidation, via erythrodiol, to oleanolic acid. In fractions II and III (Tables 4 and 5) containing both these compounds, their radioactivity was very low and it slowly rose throughout the experiment.

In fraction IV all the derivatives of oleanolic acid monoglucoside were labelled (Table 3). The radioactivity of monoglucoside **1g** decreased throughout the experiment, whereas glycosides **2g** and **3g** attained maximum labelling after 24 hr, and both pentaglycosides **6g** and **7g** were maximal after 70 hr. These results indicated that the biosynthesis of all oleanolic acid glucosides takes place in the microsomal fraction, in agreement with the earlier proposed scheme [4]. The rapid drop in the radioactivity of pentaglycosides suggested that they are most probably removed from this fraction and translocated to the other fractions.

Fraction III contained radioactive glycosides **1g**, **2g**, and **6g**. In glycoside **1g** a small decrease of radioactivity (Table 4) was observed and in the remaining glycosides continuous increases were noted. It seems that these compounds reach this fraction as a result of transport from the microsomes. However, in view of the slight drop in radioactivity in glycoside **1g** a partial transformation of glycoside **1g** to glycosides **3g** and **6g** may occur, most probably in the fragments of the Golgi apparatus. This would be analogous to the glycosylation of sterols and

Table 4. MVA-[2^{14}C] incorporation into triterpenes in subfraction III obtained from *C. officinalis* leaves*

Compound	Time			
	4 hr	24 hr	70 hr	280 hr
	cpm			
β-Amyrin	290	770	1150	1340
Erythrodiol	30	50	50	110
Oleanolic acid in glycosides				
1g	350	930	640	600
2g	40	240	180	300
3g	270	1040	2650	3750
6g	0	760	3470	4500
7g	0	0	510	440
F	810	2550	3300	5480
D	750	3410	14880	70680
C	300	4000	13850	45800
B	0	280	1200	2920
A	120	1350	6700	8550

* From 10 g of leaves.

Table 6. MVA-[2^{14}C] incorporation into triterpenes in subfraction I obtained from *C. officinalis* leaves*

Compound	Time			
	4 hr	24 hr	70 hr	280 hr
	cpm			
β-Amyrin	0	0	0	0
Erythrodiol	0	0	0	0
Oleanolic acid in glycosides				
1g	0	0	0	0
2g	0	0	0	0
3g	0	0	0	0
6g	0	0	0	0
7g	0	370	2460	10840
F	0	0	0	0
D	0	760	7510	27490
C	0	430	2810	4200
B	110	720	13400	19850
A	0	260	1950	4150

* From 10 g of leaves.

Table 7. MVA-[2¹⁴C] incorporation into triterpenes in subfraction V obtained from *C. officinalis* leaves*

Compound	Time			
	4 hr	24 hr	70 hr	280 hr
	cpm			
β-Amyrin	0	0	0	0
Erythrodiol	0	0	0	0
Oleanic acid in glycosides				
1g	160	90	60	0
2g	70	170	140	0
3g	0	0	0	0
6g	120	560	5510	23020
7g	0	230	1200	110
F	15000	3540	230	240
D	0	0	260	0
C	0	0	120	0
B	180	240	380	440
A	100	160	260	330

* From 10 g of leaves.

acylation of sterol glycosides. Perhaps this process and transport take place simultaneously.

Fraction II contained only glycoside **6g**, in which radioactivity increased throughout the experiment (Table 5). Possibly this compound reaches fraction II from the microsomes and becomes incorporated into the chloroplast structure or is absorbed onto the chloroplast envelope during the procedure of preparation.

Fraction I contained only the final product of the oleanolic acid transformations, i.e. glycoside **7g**. Its radioactivity rose throughout the experiment (Table 6) suggesting that it is selectively transported from the microsomal fraction and incorporated into the cell plasmalemma or walls.

In fraction V a continuous increase in the radioactivity of glycoside **6g** was observed; there was little radioactivity in glycoside **7g**, which occurred in only trace amounts. According to our previous findings [5], this glycoside is transported from leaves to the root. In the present investigation this transport was impossible, since the experiments were performed on shoots with cut off roots, and perhaps for this reason glycoside **6g** accumulated in the cytosol.

These results suggested that the biosynthesis of all the derivatives of oleanolic acid 3-glucoside occur in the microsomal fraction, from which they are subsequently transported to the other cell organelles. Evidently, there is some 'specialization': the final products of transformations of this series of glycosides accumulate in the cell walls and plasmalemma (glycoside **7g**) or in the mitochondria, chloroplasts and cytosol (glycoside **6g**). Different results were obtained for derivatives of oleanolic acid 3-glucuronoside.

In the microsomal fraction radioactive glycosides F, D and C were observed (Table 3). The radioactivity of monoglucuronoside decreased from the beginning of the experiment, that of diglycoside D increased till 70 hr and then abruptly dopped, whereas that of triglycoside C appeared after 24 hr and increased throughout the experiment. The results indicate that in the microsomal fraction monoglucuronoside is the first to be formed, whereupon diglycoside and one of the triglycosides are produced. The remaining radioactive glucuronosides did not occur in this fraction: it seems that they are formed in other subfractions.

In fractions III, II and I the radioactivity of glucuronosides increased throughout the experiment. The particularly high calculated specific radioactivity of glucuronoside A in the mitochondrial–Golgi fraction suggested that perhaps this fraction is the site not only of the accumulation, but also of the formation of glucuronosides A and B, most likely in the fragments of the Golgy apparatus, similar to steryl glucoside and acyl steryl glucoside [7]. In fraction V the radioactivity of monoglucuronoside dropped rapidly between the 4th and 70th hr and then remained constant, whereas that of glucuronosides A and B increased till the 70th hr to a constant level. After 24 hr radioactive glycosides D and C appeared, though neither of these compounds was demonstrated in this fraction upon quantitative determination. Perhaps the compounds occurring in the soluble fraction are transported from their biosynthesic site to the remaining organelles, where they accumulate. It is possible that glucuronosides, like the various forms of sterols, can be incorporated into the membrane structures of cell organelles. On the other hand, it also seems possible that these compounds, especially those with a greater number of sugar molecules, may accumulate in the cell vacuole, and subsequently become adsorbed on the surface of various organelles during the preparative procedure.

The present results indicate that squalene undergoes cyclization to β-amyrin in the microsomal fraction of *C. officinalis* leaves. In the same fraction β-amyrin is oxidized via erythrodiol to oleanolic acid; immediate addition of a molecule of glucuronic acid to the latter yields oleanolic acid monoglucuronoside, or addition of a glucose molecule affords oleanolic acid monoglucoside. Moreover, in the same fraction all glucosides and the first glucuronosides are synthesized. The remaining two glucuronosides are formed in other subfractions. It seems that glucuronosides occurring in *C. officinalis* during the whole vegetation period can participate, as do the various forms of sterols, in the formation of certain structures of cell organellae. On the other hand, oleanolic acid glucosides, which appear only in leaves of 30-day-old *C. officinalis* plants, play a different role. Pentaglycoside **6g** occurring in the largest amount is the form in which oleanolic acid is transported from leaves to the root and it accumulates in the cytosol. Another pentaglycoside, **7g** accumulates in the cell wall and membranous fractions.

EXPERIMENTAL

Material. MVA-[2¹⁴C] administration, preparation of the cellular subfractions and their extraction have been reported previously [7].

Preparative chromatography. Combined MeOH and Et_2O extracts were divided into two equal parts, one of which was used for isolation of squalene, β-amyrin and erythrodiol; the other part served to isolate oleanolic acid glycosides. Squalene β-amyrin and ertyhrodiol were isolated by TLC under conditions used for the various forms of sterols [8]. Subsequently, β-amyrin was purified by TLC on Al_2O_3 impregnated with rhodamine G6. 2 mg/1g Al_2O_3 in the system hexane-C_6H_6-EtOH (500:500:8) and for erythrodiol development with alcohol free $CHCl_3$. Oleanolic acid glycosides were separated by TLC on Si gel as described earlier [3, 4].

Quantitative determinations. Free β-amyrin and erythrodiol were acetylated with radioactive Ac_2O and determined quantitatively as described in ref. [7]. Oleanolic acid obtained from the

different glycosides was methylated with an excess of CH_2N_2 in Et_2O at 2° for 24 hr, whereupon to each sample 20 μg cholesterol as internal standard was added; the mixture was separated by GLC with 1% SE-30 on Chromosorb W (80–100 mesh). The amount of oleanolic acid methyl ester were calculated in relation to the amount of cholesterol.

Other methods. Hydrolysis, radioactivity measurement and protein content were carried as described previously [3, 8, 9].

REFERENCES

1. Kasprzyk, Z. and Wojciechowski, Z. (1967) *Phytochemistry* **8**, 69.
2. Miller, H. (1970) M. Sci. Thesis, University of Warsaw.
3. Kasprzyk, Z., Wojciechowski, Z. and Janiszowska, W. (1970) *Phytochemistry* **9**, 561.
4. Kasprzyk, Z., Janiszowska, W. and Sobczyk, E. (1973) *Acta Biochim. Polon.* **20**, 231.
5. Janiszowska, W. and Kasprzyk, Z. (1974) *Acta Biochim. Polon.* **21**, 415.
6. Wasilewska, A. (1970) M. Sci. Thesis, University of Warsaw.
7. Janiszowska, W. and Kasprzyk, Z. (1977) *Phytochemistry* **16**, 473.
8. Adler, G. and Kasprzyk, Z. (1975) *Phytochemistry* **14**, 723.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.