Phytochemistry Vol 22, No 1, pp 107–109, 1983
Printed in Great Britain

0031-9422/83/010107-03$03 00/0
Pergamon Press Ltd

BIOSYNTHESIS OF OLEANOLIC ACID GLYCOSIDES IN *CALENDULA OFFICINALIS* INFLORESCENCES

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(*Received* 3 *March* 1982)

Key Word Index—*Calendula officinalis*, Compositae, oleanolic acid glycosides, labelling dynamics

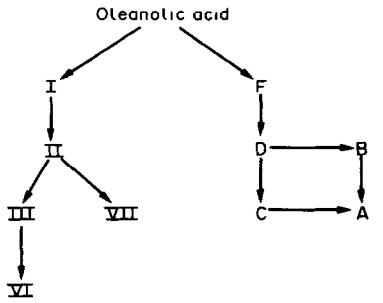
Abstract—In *Calendula officinalis* inflorescences administrated [3-^{3}H]oleanolic acid derivatives, i e the 3-O-monoglucoside and 3-O-monoglucuronide were assembled and their metabolism and transport from involucre to flowers was demonstrated Besides the effective glycosylation to derivatives of its own series, each of the administrated precursors undergoes hydrolysis to give free oleanolic acid and then glycosylation to yield derivatives of the other series

INTRODUCTION

Our earlier work reported the localization, structure and biosynthesis of oleanolic acid glycosides in *Calendula officinalis* [1–3] Two series of these compounds, namely, derivatives of oleanolic acid 3-O-glucoside (I) and oleanolic acid 3-O-glucuronide (F) have been found In the shoots and flowers the following compounds are present 3-O-[gal(1→4)glc]-oleanolic acid (II), 3-O-[gal–gal(1→4)glc]-oleanolic acid (III), 3-O-[gal–gal(1→4)glc-1, glc–glc(1→3)glc-1]-oleanolic acid (VI), 3-O-[gal(1→4)glc-1, glc–glc–glc(1→3)glc-1]-oleanolic acid (VII), 3-O-[gal(1→3)glur]-oleanolic acid (D), 3-O-[gal(1→3)glur]-28-O-glc-oleanolic acid (C), 3-O-[glc(1→4)glur, gal(1→3)glur]-oleanolic acid (B) and 3-O-[gal(1→4)glur, gal(1→3)glur]-28-O-glc-oleanolic acid (A)

Using [1-^{14}C]acetate[2] and $^{14}CO_2$[3] the sequence of biosynthesis of both series of glycosides in shoots was investigated

Since the largest quantity of oleanolic acid glycosides accumulates in ligulate flowers (2% of oleanolic acid by dry wt) an investigation of their biosynthesis in inflorescences was undertaken using as precursors the radioactive oleanolic acid monoglycosides belonging to both series, i.e 3-O-glucoside and 3-O-glucuronide



Scheme 1

RESULTS AND DISCUSSION

The radioactive precursors were administrated to marigold inflorescences and subsequently the radioactivity was determined in the free oleanolic acid, the unused precursor and the total glycosides of each series Radioactive oleanolic acid 3-O-glucoside was administrated for 4, 6 and 8 hr and after the selected time the flowers and involucre were analysed separately The mixture of glycosides was separated by TLC, yielding in order of increasing polarity free oleanolic acid, glycosides of series II, glycosides VII and F and glycosides of series I Subsequently glycosides VII and F were separated (solvent system. ethyl acetate–acetic acid–water, 3 3 1) and added to the compounds of the respective series The radioactivity incorporated after administration of the 3-O-glucoside is presented in Table 1

Radioactivity was found not only in administrated precursor and its derivatives, i.e glycosides of series II, but also in free oleanolic acid and in glycosides of series I. This result indicated that glucoside I was not only glycosylated to yield derivatives of series II but it was also hydrolysed to give free oleanolic acid and then transformed to glycosides of series I The total radioactivity of oleanolic acid derivatives incorporated into involucres was at a maximum after 4 hr and then decreased This result suggests that a radioactive precursor absorbed by the inflorescence is transported from the involucre to the flowers In the flowers total radioactivity increased continually up to the end of the experiment attaining, after 8 hr, 74% of the total radioactivity

In the involucre radioactivity of glucoside I precursor and its derivatives also attained a maximal level after 4 hr. Radioactivity in oleanolic acid reached a peak after 6 hr, which suggested that the process of hydrolysis of glucoside I was delayed in relation to the process of its glycosylation The presence of radioactivity in glucuronides after 4 hr suggested the glucuronidation of free oleanolic acid The further decrease of their radioactivity in-

Table 1 Incorporation of oleanolic acid 3-O-glucoside (I) into compounds by the inflorescences of *Calendula officinalis*

	Time of incubation (hr)					
	4		6		8	
Compound	inv *	fl	inv	fl	inv	fl
Free oleanolic acid	35 2†	22 1	35 4	19 0	6 2	7 1
Glucoside I	27 4	4 7	7 2	19 9	5 7	21 9
Glycosides of series II	79 0	16 6	17 3	13 8	4 9	19 7
Glycosides of series I	6 9	3 1	14 0	8 0	1 6	2 4

*inv , Involucre, fl , flowers
†nMol oleanolic acid/g fr wt

dicated the possibility of transport of these compounds into the flowers

In flowers the radioactivity of glucoside I continually increased, thus suggesting its active transport from the involucre The radioactivity present in its derivatives (series II), also increased and at the start of the experiment was higher than that of the precursor These results indicate that glycosides of series II may be the transport form from involucre to flowers The radioactivity in free oleanolic acid decreased continually during the experiment but the labelling of the glucuronides reached a peak after 6 hr. On the basis of these results it is difficult to decide whether free oleanolic acid is transported from the involucre to the flowers or if it is formed in the flowers as a consequence of hydrolysis of glucoside I

The dynamics of incorporation of radioactivity into different glycosides fractions, using 3-O-glucuronide (F) as the precursor, was investigated after 2, 4 and 8 hr In contrast to the previous results with the 3-O-glucoside as the precursor, the first sampling time was at 2 hr and the 6 hr sample was omitted The results are presented in Table 2

After administrating the 3-O-glucuronide a continous increase in radioactivity in both parts of the inflorescences was observed However the radioactivity incorporated into the flowers was lower than that incorporated into the involucre in the same time In the involucre, radioactivity incorporated as glucuronide F after 2 hr, was present not only in this compound and in other glycosides of series I, but also in glycosides of series II and particularly in free oleanolic acid (nearly 40% of the total) This indicated that in the involucre glucuronide F was hydrolysed better than glycoside I and that the process of hydrolysis of glucuronide F dominated the process of its glycosylation to the glycosides of series I

Radioactivity incorporated into free oleanolic acid reached a peak at 4 hr and subsequently decreased, probably as the result of its transformation into glycosides of both series in which the radioactivity continually increased Radioactivity in the administrated glycoside F decreased at the beginning of the experiment presumably as the result of increased hydrolysis It then rapidly increased, probably as a result of a slowing down of the processes of hydrolysis and glycosylation This result shows that the rate of hydrolysis of the 3-O-glucuronide is especially higher at the beginning of the experiment

In the flowers, after administration of glycoside F to the inflorescences most of the radioactivity (85%) was in free oleanolic acid after 2 hr, whereas the total amount of radioactivity in both series of glycosides was low This result suggested that oleanolic acid can be transported from the involucre to the flowers as the free compound and then transformed into glycosides of both series in the flowers This conclusion was also supported by the increased level of radioactivity observed in oleanolic acid It was interesting to note that in the involucre as well as in the flowers a delay occurred in the biosynthesis of glycosides of

Table 2 Incorporation of oleanolic acid 3-O-glucuronide into compounds by inflorescences of *Calendula officinalis*

	Time of incubation (hr)					
	2		4		8	
Compound	inv *	fl	inv	fl	inv	fl
Free oleanolic acid	7 6†	4 5	13 3	0 6	8 2	0 9
Glucuronide F	4 6	0 3	3 6	0 3	14 0	1 6
Glycosides of series I	4 8	0 1	5 5	0 6	12 2	0 6
Glycosides of series II	3 2	0 4	10 9	1 1	20 4	3 5

* inv , Involucre, fl , flowers
† nMol oleanolic acid/g fr wt

series I in comparison to glycosides of series II, in spite of administration of the direct precursor glucuronide of glycosides of series I This result did not indicate the transport of glucuronide F from involucre to flowers and did not exclude the transport of glycosides of series II

Analysis and comparison of the results of incorporation of 3-O-monoglucoside (I) and 3-O-monoglucuronide (F) by marigold inflorescences indicate the following conclusions

The 3-O-glucoside is absorbed and metabolized in inflorescences better than the 3-O-glucuronide After administration of both precursors to inflorescences the incorporation of radioactivity into free oleanolic acid and into glycosides of both series was observed in involucres as well as in the flowers Glucoside I is primarily glycosylated to derivatives of its series and to a lesser extent hydrolysed to free oleanolic acid in involucres and flowers Glucuronide F is more effectively hydrolysed to free oleanolic acid than glycosylated to derivatives of its series In addition to effective glycosylation to derivatives of its series each of the administrated precursors undergoes glycosylation to derivatives of the opposite series This indicates that formation of the opposite series of glycosides proceeds after hydrolysis of precursor to free oleanolic acid Radioactive compounds are translocated from involucre to flowers The transport form can be free oleanolic acid or glucosides of series II.

EXPERIMENTAL

Preparation of radioactive precursors [3-^{3}H]Oleanolic acid was obtained by reduction of 3-keto oleanolic acid with NaB^3H_4 The 3-O-glucoside was synthesized by reaction of tetra-acetylbromide glucose with radioactive oleanolic acid[4], whereas the 3-O-glucuronide was obtained by reaction of the acetylbromide of glucuronic acid with radioactive oleabolic acid using slightly modified conditions The 3-O-glucoside had a sp act of 2.5×10^5 cpm/mg and the 3-O-glucuronide had a sp act of 9×10^5 cpm/mg

Administration of radioactive precursors and fractionation of plant material The soln of radioactive precursor in 5% EtOH–H_2O was administrated to marigold inflorescences through the stems after 2, 4, 6 and 8 hr Subsequently inflorescences were separated into involucres and flowers and each part was analysed separately Individual parts were ground with dry Na_2SO_4 and extracted twice with cold MeOH and twice with hot MeOH To the methoanolic extract one-third vol of H_2O was added, the MeOH was distilled and the aq residue was extracted $\times 4$ with *n*-BuOH to obtain the crude fraction of glycosides

Preparative chromatography The crude glycosides were separated by TLC on Kieselgel G-60 (Merck) with the solvent system $CHCl_3$–MeOH–H_2O (61 32 7) Individual compounds were purified in systems described earlier[3]

Radioactivity measurement Radioactivity of eluted compounds was measured in toluene-based scintillator at an efficiency of 48%

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