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BIOTRANSFORMATION OF PROGESTERONE BY SUSPENSION CULTURES OF *DIGITALIS PURPUREA* CULTURED CELLS*

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Key Word Index—*Digitalis purpurea*; Scrophulariaceae; tissue cultures; biotransformation; glucosidation; steroid; progesterone; 5α-pregnan-3β-ol-20-one glucoside; 5α-pregnane-3β, 20α-diol glucoside; 5α-pregnane-3β,20β-diol glucoside; Δ⁴-pregnen-20α-ol-3-one glucoside; Δ⁴-pregnen-20β-ol-3-one glucoside.

Abstract—It has been shown that the cultured cells of *Digitalis purpruea* are capable of transforming progesterone (I) to 5α-pregnane-3,20-dione (II), 5α-pregnan-3β-ol-20-one (III), its glucoside (IV), 5α-pregnane-3β,20α-diol (V), its glucoside (VI), 5α-pregnane-3β,20β-diol (VII), its glucoside (VIII), Δ⁴-pregnen-20α-ol-3-one (IX), its glucoside (X), Δ⁴-pregnen-20β-ol-3-one (XI) and its glucoside (XII). 5α-Pregnan-3β-ol-20-one glucoside (IV), 5α-pregnane-3β,20α-diol glucoside (VI), 5α-pregnane-3β,20β-diol glucoside (VIII), Δ⁴-pregnen-20α-ol-3-one glucoside (X) and Δ⁴-pregnen-20β-ol-3-one glucoside (XII) have been found for the first time as new metabolites by plant tissue cultures. A scheme for the biotransformation of progesterone (I) has been proposed, and the reduction and glucosidation activities distinctly have been observed in these cultured cells.

INTRODUCTION

IN AN earlier publication, we reported the conversion of progesterone[1] (I) into 5α-pregnan-3β-ol-20-one (III) and its palmitate in suspension cultures of *Nicotiana tabacum* and *Sophora angustifolia* cultured cells and digitoxin[2] into purpurea glycoside *A*, *B* and gitoxin in *Digitalis purpurea* suspension cultured cells.

It has been observed by many workers that (I) can be readily transformed to (II) and (III) by *Digitalis purpurea* and other plant suspension cultures,[3] and also to (II) by microsomes from *Dioscorea deltoidea* and *Cheiranthus cheiri* cultured cells.[4] Recently, the transformation of (I) to (III) and (VII), which are present in conjugated forms, has been reported for *Dioscorea deltoidea* suspension cultures.[5]

In order to investigate cardenolide production by plant tissue cultures, we have examined the transformation of progesterone (I) and other key intermediates in cardenolides biosynthesis by *Digitalis* plant suspension cultures. We now wish to report that (I) is transformed by suspension cultures of *Digitalis purpurea* cultured cells into 5α-pregnane-3,20-dione (II), 5α-pregnan-3β-ol-20-one (III) and its glucoside (IV) and four other metabolites

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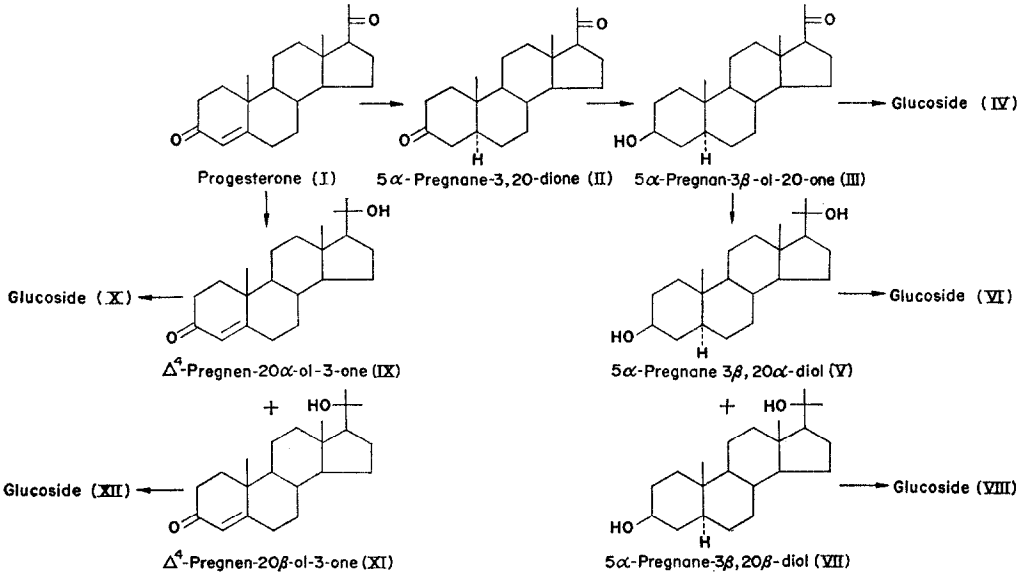
and their glucosides; 5α-pregnane-3β,20α-diol (V) and its glucoside (VI), 5α-pregnane-3β,20β-diol (VII) and its glucoside (VIII), Δ^4-pregnen-20α-ol-3-one (IX) and its glucoside (X), and Δ^4-pregnen-20β-ol-3-one (XI) and its glucoside (XII) as shown in Scheme 1. The glucosides are all new compounds.



SCHEME 1. BIOTRANSFORMATION OF PROGESTERONE BY SUSPENSION CULTURES OF *Digitalis purpurea* CULTURED CELLS.

RESULTS

[4-^{14}C]Progesterone (0·01 mCi) was incubated with *Digitalis purpurea* suspension callus culture for 3 days. The callus and medium were extracted with *n*-hexane, $CHCl_3$ and $CHCl_3$–MeOH (2:1). Approximately 40% of original radioactivity was recovered, the distribution in each fraction is given in Table 1. It was shown by TLC that most radioactivity was present in more polar substances, which were isolated from callus tissues to which cold progesterone (total 540 mg) had been administered. After 10 days, the $CHCl_3$ and $CHCl_3$–MeOH fractions were obtained from the callus and medium, respectively as before. The presence of several transformation products such as 5α-pregnane-3,20-dione (II; R_f 0·60), 5α-pregnan-3β-ol-20-one (III; R_f 0·34), 5α-pregnane-3β,20α-diol (V; R_f 0·17), 5α-pregnane-3β,20β-diol (VII; R_f 0·17), Δ^4-pregnen-20α-ol-3-one (IX; R_f 0·24) and Δ^4-pregnen-20β-ol-3-one (XI; R_f 0·24) were detected in the $CHCl_3$ fraction of the medium by TLC ($CHCl_3$–EtOAc 4:1) and GLC after separation with preparative TLC (see Experimental). At the same time (III) was isolated as colorless leaflets, m.p. 188–9°.

The $CHCl_3$ and $CHCl_3$–MeOH fractions of the callus and the $CHCl_3$–MeOH fraction of the medium were chromatographed on silica gel to give six transformation products after co-column chromatography and preparative TLC. 5α-Pregnan-3β-ol-20-one (III) was again obtained crystalline, and its identity confirmed by m.m.p., IR, TLC and GLC with an authentic sample. The more polar transformation products, which were considered to be conjugates (R_f 0·58 and 0·51; $CHCl_3$–MeOH, 3:1) agreed with those found in the radioactive fractions above, were isolated as acetates (R_f 0·55, 0·50 and 0·35; $CHCl_3$–EtOAc, 4:1).

The glucoside (IV) was isolated from the acetate mixture as colorless needles, m.p. 196·5–7°. NMR analysis of the acetate of (IV) showed an anomeric proton doublet at $\delta = 4{\cdot}55$ (1H) with coupling of 7·5 Hz. The coupling constant of the anomeric doublet indicates that sugar has the β-configuration. The MS is consistent with (IV) acetate being a hexopyranoside tetraacetate.[6] In none of spectra was a molecular peak observed, but peaks were found at *m/e* 588 (2·2%) [M^+–AcOH][7] with the primary fission the base peak fragment at 301 (aglycone). Furthermore, peaks diagnostic of a hexopyranoside tetraacetate were observed at *m/e* 331 (5%), tetraacetyl glucose oxonium ion. Acid hydrolysis of the acetate of (IV) gave 5α-pregnan-3β-ol-20-one (III) and glucose as the sole sugar by TLC. Therefore, this acetate was 5α-pregnan-3β-ol-20-one (III) tetraacetyl glucopyranoside and this was confirmed by m.m.p., IR, MS and NMR with synthetic 20-oxo-5α-pregnan-3β-yl 2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranoside.

TABLE 1. DISTRIBUTION OF ^{14}C AFTER INCUBATING [4-^{14}C]PROGESTERONE WITH *Digitalis purpurea* SUSPENSION CULTURES FOR 3 DAYS

	Fraction	Total activity dpm × 10^{-6}	(%)		Fraction	Total activity dpm × 10^{-6}	(%)
Callus	*n*-Hexane	1·22	5·5	Medium	$CHCl_3$	1·17	5·3
	$CHCl_3$	2·32	10·5		$CHCl_3$–MeOH	0·33	1·5
	$CHCl_3$–MeOH	3·89	17·5			(Total	40·3)

The mixture containing the acetates of (X) and (XII) was purified by preparative TLC (R_f 0·35; $CHCl_3$–EtOAc, 4:1) and their structures were elucidated by the combination instrument GC–MS and acid hydrolysis. In the MS of (X) acetate with GC–MS (R_t 14·8 min) the peaks with fragmentation diagnostic of hexopyranoside tetraacetate were observed at *m/e* 646 (1%) [M^+], 331 (22) and 299 (100) (aglycone). Similarly, (XII) acetate (R_t 9·9 min) gave *m/e* 646 (1·2), 331 (30) and 299 (100). By acid hydrolysis the mixture gave Δ^4-pregnen-20α-ol-3-one (IX) and Δ^4-pregnen-20β-ol-3-one (XI) as aglycones and glucose. Therefore, the structures of (X) and (XII) were considered to be Δ^4-pregnen-20α-ol-3-one glucoside and Δ^4-pregnen-20β-ol-3-one glucoside, respectively.

Finally, the mixture containing the acetates of (VI) and (VIII) was obtained as colorless leaflets, whose main peaks were observed at *m/e* 632 (2·0%) [M^+–AcOH], 345 (5) (aglycone), 331 (28) and 285 (100) in the MS. By acid hydrolysis the mixture gave 5α-pregnane-3β,20α-diol (V), 5α-pregnane-3β,20β-diol (VII) and glucose. The structures of (VI) and (VIII) were therefore presumed to be 5α-pregnane-3β,20α-diol glucoside and 5α-pregnane-3β,20β-diol glucoside, respectively.

DISCUSSION

As shown in Scheme 1, the formation of 5α-pregnan-3β-ol-20-one glucoside (IV) could be explained by the stereospecific reduction of an α,β-unsaturated ketone to 5α-pregnan-3β-ol-20-one (III) via 5α-pregnane-3,30-dione (II) followed by glucosidation. Also 5α-pregnane-3β,20α-diol (V), 5α-pregnane-3β,20β-diol (VII), and Δ^4-pregnen-20α-ol-3-one (IX), Δ^4-pregnen-20β-ol-3-one (XI), could be formed by reduction of the 20-ketone group of (III)

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and (I) respectively, and are then transformed into their glucosides; (VI), (VIII), (X) and (XII). The reduction of (I) → (II) → (III) is well known both in animals and plants,[8] including plant tissue cultures of several species such as *Digitalis purpurea*,[3] microsomes from *Dioscorea deltoidea* and *Cheiranthus cheiri* suspension cultures,[4] *Nicotiana tabacum* and *Sophora angustifolia* tissue cultures,[1] and leaf homogenates of cardenolide producing plants.[9]

However, it is of great interest that the palmitate of (III) in *Nicotiana* and *Sophora* tissue cultures[1] was not detected in *Digitallis* tissue culture, but (IV), (VI), (VIII), (X) and (XII), were found instead. The first step in the biosynthesis of cardenolides in the *Digitalis* plant[10] is the reduction of (I) to 5β-pregnane-3,20-dione, but only 5α-compounds were found in metabolic products from the callus. This suggests that the stereospecific reduction (Δ^4 to *A/B cis*) must be under strict enzymatic control in *Digitalis purpurea* tissue culture. The conversion of cholesterol to yonogenin and tokorogenin of the *A/B cis* type has been reported in *Dioscorea tokoro* tissue culture[11] and the presence of a cholesterol side-chain cleaving enzyme has been demonstrated in seedlings of *Digitalis purpurea* but not in tissue cultures of *Digitalis purpurea*.[12]

It is not known in *Digitalis purpurea* tissue culture whether the reduction of (III) to (V) and (VII) is catalysed by a 20α- or 20β-hydroxysteroid dehydrogenase[5] as is (I) to (IX) and (XI). Microbial reduction of 20-ketosteroids mostly yields 20β-alcohols, but 20α-reduction as well as the formation of both epimers has also been reported.[13–15] The 20α-compounds (V, IX) were detected by GLC analysis in high amounts than the corresponding 20β-compounds (VII, XI), and it thus seems that the activity of 20α-hydroxysteroid dehydrogenase may be higher in *Digitalis purpurea* tissue culture.

Some naturally occurring steroids are naturally present as glycosides in higher plants and moreover it has been reported that several steroids, including (III), were converted into β-D-glucopyranoside by slices of potato tuber (*Solanum tuberosum*).[16] However, the acetate of (IV) was isolated in crystalline form for the first time allowing us to determine its structure, and (VI), (VIII), (X) and (XII), are new compounds. We suggest that the conjugated metabolites, which Stohs *et al.* have observed after the metabolism of progesterone,[5] cholesterol[17] and Δ^4-androstene-3,17-dione[18] by *Dioscorea deltoidea* tissue cultures, are probable glucosides like (IV), (VI), (VIII), (X) and (XII).

The *Digitalis purpurea* tissue culture we examined, maintained the same biosynthetic potential regarding the conversion of digitoxin into purpurea glycosides *A* and *B* and into gitoxin as the intact plant, but did not convert progesterone into the cardenolides. The efficient production of these compounds by *Digitalis* tissue culture is not yet realized and will be the object of future investigations.

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EXPERIMENTAL

NMR spectra were determined in $CDCl_3$ using tetramethylsilane as internal reference. MS were run, using a direct insertion probe, on an JMS-OIS instrument (source 95–160°, 75 eV ionizing energy).

Tissue culture and progesterone administration. The tissue cultures used were derived from seedling of *Digitalis purpurea,* sub-cultured for about 4 yr. Plant tissue suspension culture was grown on modified Murashige and Skoog's tobacco medium containing 0·5 ppm 2,4-dichlorophenoxyacetic acid and 0·1 ppm kinetin. The medium (250 ml) was dispensed in 1 l. flask containing 0·01 mCi [4-^{14}C]progesterone (29·3 mCi/mmol, Daiichi Pure Chemicals Co., Ltd.) in C_6H_6 (30 mg progesterone in the cold experiment). The callus (25–35 g) from 4 weeks static cultures were incubated at 29° in a shaker for 3 days (10 days in the cold experiment).

Extraction procedure. The callus and medium were separated through Nylon cloth and the medium extracted with $CHCl_3$ and then $CHCl_3$–MeOH (2:1). The callus was homogenized and extracted under reflux with MeOH × 3, the filtrates combined, and MeOH evaporated under vacuum. The residue was extracted 3 × equal vol. of *n*-hexane, $CHCl_3$ and $CHCl_3$–MeOH (2:1), respectively. Each fraction was evaporated for scintillation (PPO and POPOP in toluene). In the same way, after administration of cold progesterone (540 mg) to *Digitalis* callus (750·4 g fr. wt), the fractions of callus, $CHCl_3$ (Fr. *A*) and $CHCl_3$–MeOH (Fr. *B*), and of medium, $CHCl_3$ (Fr. *C*) and $CHCl_3$–MeOH (Fr. *D*) were obtained for the isolation and identification of (II) to (XII).

Detection of 5α-*pregnane*-3,20-*dione* (II), 5α-*pregnan*-3β-*ol*-20-*one* (III), 5α-*pregnane*-3β,20α-*diol* (V), 5α-*pregnane*-3β,20β-*diol* (VII), Δ^4-*pregnen*-20α-ol-3-*one* (IX) *and* Δ^4-*pregnen*-20β-*ol*-3-*one* (XI). Fr. *C* was investigated by TLC with authentic compounds. After separation on preparative TLC ($CHCl_3$–EtOAc, 4:1), several transformation products were identified by GLC. R_f and R_t are as follows: (II), R_f 0·60, R_t min 21·40; (III), 0·34, 14·80; (V), 0·17, 10·60; (VII), 0·17, 9·30; (IX), 0·24, 33·20; (XI), 0·24, 28·10.

Conditions of GLC. A glass U-column (1·8 m × 4 mm i.d.) packed with 1% QF-1 on Gas Chrom Q (80–100 mesh) was operated under the following conditions: detector block and column oven were at 270° [250° in (II)] and 220° [200° in (II)], respectively. The carrier gas was N_2 with a flow rate of 76 ml/min [72° in (II)], with a F.I.D.

Isolation and identification of 5α-*pregnan*-3β-*ol*-20-*one* (III), 5α-*pregnan*-3β-*ol*-20-*one glucoside* (IV), 5α-*pregnane*-3β,20α-*diol glucoside* (VI), 5α-*pregnane*-3β,20β-*diol glucoside* (VIII), Δ^4-*pregnen*-20α-*ol*-3-*one glucoside* (X) *and* Δ^4-*pregnen*-20β-*ol*-3-*one glucoside* (XII). Fr. *A*, *B* and *D* (total 2·22 g) were chromatographed on silica gel (300 g) and eluted as follows: fraction *a* (Fr. *a*), $CHCl_3$ 4·7 l.; *b*, 5% MeOH in $CHCl_3$ 11 l.; *c*, 10% MeOH in $CHCl_3$ 2 l.; *d*, 20% MeOH in $CHCl_3$ 1·5 l. and *e*, MeOH 1 l. (III) from Fr. *a* was recrystallized from MeOH–H_2O after purification on preparative TLC ($CHCl_3$–EtOAc, 4:1) several times. Colorless leaflet (38·5 mg), m.p. 188–9°, was identified by TLC, GLC, IR and m.p. comparison with authentic sample. The IR, ν_{max}^{KBr} cm^{-1} 3420, 3370 (ν O–H), 2900, 2820 (ν C–H), 1697, 1680 (ν C=O), 1040 (ν C–O). By a similar method 34·3 mg (III) was also obtained from Fr. *C*. From Fr. *b*, the brown oil was acetylated giving the acetates (108·7 mg) of (IV), (X) and (XII); 76·6 mg: (VI) and (VIII) (Ac_2O–pyridine). The acetates were separated and purified on preparative TLC (R_f 0·50, $CHCl_3$–EtOAc, 4:1) several times. (IV) acetate was recrystallized from MeOH to yield colorless needles (13·4 mg), m.p. 196·5–7° (Found: C, 64·53; H, 8·22. $C_{35}H_{52}O_{11}$ requires: C, 64·78; H, 8·09%.) IR, ν_{max}^{KBr} cm^{-1} 2940, 2850 (ν C–H), 1750, 1685 (ν C=O), 1370, 1245, 1230, 1040 (ν C–O–C). NMR (δ, ppm); 0·60 (C-18, S, 3H), 0·78 (C-19, *S*, 3H), 1·97, 1·98, 2·00, 2·05, 2·07 (C-21, *S*, 3H and Ac, *S*, 4 × 3H), 4·55 (1'-H, *d*, 1H, J=7·5 Hz). MS, *m/e* 588 (2·2%) [M$^+$–AcOH], $C_{33}H_{48}O_9$ (required 588·329) 588·328, 331 (5), 301 (100), 242 (30), 200 (12), 169 (20), 157 (20), 145 (12), 140 (12), 115 (12), 109 (23), 98 (15), 73 (5) and 43 (97). The mixture containing (X) and (XII), as acetates was obtained by preparative TLC (R_f 0·35; $CHCl_3$–EtOAc, 4:1). MS of (X) with GC–MS (R_t 14·8 min); *m/e* 646 (1%) [M$^+$], 331 (22), 299 (100), 281 (20), 269 (20), 242 (8), 200 (7), 169 (72), 157 (12), 140 (7), 115 (10), 109 (30), 98 (13), 73 (5) and 43 (47). MS of (XII) with GC–MS (R_t 9·9 min); *m/e* 646 (1·2) [M$^+$], 331 (30), 299 (92), 281 (25), 269 (25), 242 (20), 200 (15), 169 (63), 157 (25), 140 (20), 115 (10), 109 (33), 98 (25), 73 (20), 43 (100). The mixture containing acetates of (VI) and (VIII), (24·35 mg) was purified by preparative TLC (R_f 0·55; $CHCl_3$–EtOAc, 4:1) after acetylation. IR, ν_{max}^{KBr} cm^{-1} 2925, 2860 (ν C–H), 1750 (ν C=O), 1365, 1230, 1035 (ν C–O–C). MS, *m/e* 632 (2·0%) [M$^+$–AcOH], 345 (5), 331 (28), 285 (100), 269 (40), 255 (43), 242 (35), 211 (5), 200 (20), 169 (77), 157 (25), 140 (18), 115 (12), 109 (40), 98 (25), 73 (5), 43 (58).

Operating conditions of GC–MS analysis. The mixture containing acetates of (X) and (XII) was run on a Shimadzu LKB-9000S instrument. A glass column (0·35 m × 3 mm i.d.) packed with 1·5% OV17 on Chromosorb W (80–100 mesh) was operated under the following conditions: column oven, injection port and ion source were 280°, 320° and 310°, respectively; carrier gas, He at 29 ml/min. atm., ionizing energy, 70 eV.

Hydrolysis of 5α-*pregnan*-3β-*ol*-20-*one glucoside acetate.* (IV) acetate (5·0 mg) from *Digitalis purpurea* callus was refluxed with 5 ml of 10% alcoholic HCl for 1 hr. After diluting with 30 ml H_2O, EtOH was removed under reduced pressure. The solution was extracted with $CHCl_3$ and evaporation of the $CHCl_3$ afforded a crystalline residue which was identified by TLC (R_f 0·34; $CHCl_3$–EtOAc, 4:1) and GLC (R_t 14·80 min). The acidic solution after removing 5α-pregnan-3β-ol-20-one was neutralized with dil. KOH and

concentrated under reduced pressure. The sugar was the same as that from the hydrolysate of authentic β-D-glucose pentaacetate by TLC (R_f 0·47 and 0·34; BuOH–AcOH–H_2O, 5:1:1). By the same method, the mixture of acetates of (VI) and (VIII) gave 5α-pregnane-3β,20α-diol, 5α-pregnane-3β, 20β-diol (R_f 0·30, 0·35, respectively, *n*-heptane–EtOAc, 5:2, developed 7 ×) and the same hydrolysate of β-D-glucose pentaacetate (R_f 0·47 and 0·34; BuOH–AcOH–H_2O, 5:1:1). The mixture containing (X) and (XII) gave Δ^4-pregnen-20α-ol-3-one, Δ^4-pregnen-20β-ol-3-one (R_f 0·74, 0·79; EtOAc–cyclohexane–*n*-heptane, 6:4:1, developed 3 ×) and the same hydrolysate of β-D-glucose pentaacetate (R_f 0·47 and 0·34; BuOH–AcOH–H_2O, 5:1:1).

Synthesis of 20-*oxo*-5α-*pregnan*-3β-*yl* 2′,3′,4′,6′-*tetra*-*O*-*acetyl*-β-*D*-*glucopyranoside* (IV).[19] Acetobromglucose (3·6 g), m.p. 88·5–90° in dried C_6H_6 (50 ml) was dropped slowly into a solution of 5α-pregnan-3β-ol-20-one (1·1 g), m.p. 190° (prepared by catalytic reduction (Pd–C) of pregnenolone) Ag_2CO_3 (1·6 g) in dried C_6H_6 under reflux. After reflux for 1 hr Ag salt was filtered and the filtrate was kept at 0° overnight. The filtrate was evaporated under reduced pressure and the residue was chromatographed on silica gel (50 g). The $CHCl_3$ eluate gave colorless needles (106·6 mg) from MeOH, m.p. 196·5–197° (Found: C64·69; H, 8·05. $C_{35}H_{52}O_{11}$ required: C, 64·78; H, 8·09%.) R_f 0·50 (TLC, $CHCl_3$–EtOAc, 4:1). The IR, NMR and MS were identical with those of (IV).

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