Phytochemistry, Vol. 25, No. 10, pp. 2420–2422, 1986.
Printed in Great Britain.

0031–9422/86 $3.00 + 0.00
Pergamon Journals Ltd.

THE QUANTITATIVE ISOLATION AND ANTIMICROBIAL ACTIVITY OF THE AGLYCONE OF AUCUBIN

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(*Received* 21 *June* 1985)

Key Word Index—Aucubin; aucubigenin; iridoid aglycones; aglycone extraction; ^{1}H and ^{13}C NMR; antimicrobial activity.

Abstract—Despite the reported instability of iridoid aglycones, a new procedure was developed for the quantitative extraction of aucubigenin from an enzymatic hydrolysate of aucubin. The aglycone was obtained in crystalline form and was for the first time spectroscopically characterized (^{1}H and ^{13}C NMR spectra). Its antimicrobial activity against yeasts, bacteria and moulds was also accurately tested.

INTRODUCTION

We [1, 2] and other workers have shown an increasing interest in the utilization of iridoid glucosides, particularly aucubin (**1**) [3–6], the most widespread and abundant (> 2% in *Aucuba japonica* [7]) iridoid glucoside, as chiral starting materials for the synthesis of prostanoid synthons.

Iridoid aglycones have always been regarded as unstable compounds and this belief has thwarted their routine isolation [8]. Nevertheless we have described the isolation of aucubigenin (**2**) [9, 10] through enzymatic hydrolysis of **1** with β-glucosidase followed by a non-quantitative and tedious manual procedure of extraction with ethyl acetate (yield *ca* 65%). The aglycone (**2**) obtained by this procedure was stable at low temperature (−20°) in ethyl acetate and was sufficiently stable at room temperature to permit its rapid chromatographic purification and the recording of an approximate ^{1}H NMR spectrum [9]. However, a satisfactory spectral characterization of **2** was achieved only for its tri-*O*-acetyl derivative [9].

This paper reports an improved procedure for the quantitative extraction of **2** and its isolation in crystalline form, with consequent acquisition of excellent and definitive ^{1}H and ^{13}C NMR data. The availability of pure **2** allowed us to establish the antimicrobial activity of this aglycone in a more reliable and reproducible way than previously reported [11–15].

RESULTS AND DISCUSSION

During our studies on the utilization of iridoid glucosides and aglycones as starting materials for new synthetic purposes we noticed to our surprise that **2** was stable in boiling ethyl acetate (bp 78°) and this suggested that it could be extracted from an enzymatic hydrolysis mixture by means of a liquid–liquid extractor. The extraction of the aglycone from the hydrolysis mixture (see Experimental) was complete in about 6 hr. The colourless extract contained chromatographically pure **2**, which was obtained, after evaporation *in vacuo* of the solvent, in a very high yield (> 95%), as an amorphous residue. For analytical purposes part of this residue was chromatographed on '*washed*' silica gel (see Experimental); crystalline **2** (mp 110°) separated spontaneously from the chromatographic fractions and remained stable indefi-

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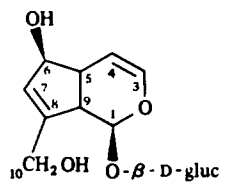

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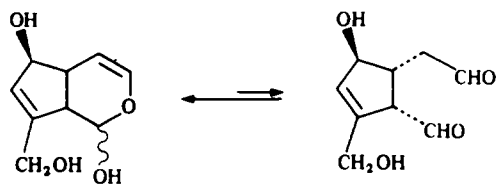

2

nitely at 0–5°. The availability of this highly purified sample of **2** and its unforeseen stability allowed us to obtain an excellent ^{1}H NMR spectrum and to record, for the first time, its ^{13}C NMR spectrum.

The ^{1}H NMR spectrum of **2** in D_2O (Table 1) was notably similar to that of the parent glucoside (**1**), except that the H-1 and H-9 resonances were closer together in the aglycone. The assignments of the signals led to the correction of some of the previous tentative assignments [9]. In addition, the integrated values for H-1 showed clearly that the D_2O solution of aucubigenin was made up at equilibrium of a mixture of β and α epimers at C-1 in a ratio of *ca* 6:1.

The anomeric equilibrium of aucubigenin was confirmed by the ^{13}C NMR spectrum (Table 2) which showed distinct resonances for most of the corresponding carbons of the β and α anomers, the β signals being found downfield of the α signals. In agreement with our recent work on the iridoid aglycones of *Deutzia scabra* [16], the anomeric C-1 of the α-form of **2** was shielded (92.20 ppm) with respect to the β-form (95.31 ppm) and the shift difference (3.11 ppm) observed between the anomeric carbons was in agreement with the corresponding $\Delta\ \delta$ values (4.08 and 3.14 ppm) reported in [16]. As expected the C-1 of the parent glucoside **1** was deshielded ('*glucosidation shift*') with respect to both anomeric carbons of β and α aglycones.

The availability of aucubigenin in pure crystalline form prompted us to verify in a definitive and reproducible way the antimicrobial properties of this aglycone, previously the subject of not completely satisfactory reports [11–15]. Unlike other authors [11, 15] we have evaluated the activity of **2** using reference strains belonging to international collections which are widely used in pharmaceutical product control [18, 19]. The activity was graded according to the diameter of zones of growth inhibition formed on agar media around paper discs containing serial dilutions of **2**. At the end of the incubation period we noted that around the paper discs a soluble dark colour had diffused into the medium, probably due to modifications of the aglycone in acidic or alkaline conditions. However this fact had no effect on the microbial activity. Aucubigenin (**2**) was active only against moulds and bacteria but not against yeasts (Table 3).

Table 1. ^{1}H NMR data for compounds **1** and **2** [300 MHz, D_2O, δ (ref. locked on HDO, δ4.70)]

		2	
H	**1**	α	β
1	5.16 *d*	5.30 *d*	4.71*d**
3	6.18 *dd*	6.16 *dd*	6.23 *dd*
4	4.98 *dd*	†	5.03 *dd*
5	2.67 *s (br)*	†	2.56 *m* (complex)
6	4.65 *dd*‡	4.47 *sext*	4.42 *sext*
7	5.73 *s (br)*	5.67 *s (br)*‡§	5.69 *s (br)*§
9	3.03 pseudo-*t*	†	2.73 *t (br)*
10 (2H)	4.18 *dd*	†	4.15 *dd*

J (Hz). **1**: $J_{1,9} = 5.0$, $J_{3,4} = 6.0$, $J_{3,5} = 1.5$, $J_{4,5} = 3.7$, $J_{10_A,10_B} = 15.0$; **2**-α: $J_{1,9} = 3.3$; **2**-β: $J_{1,9} = 6.7$, $J_{3,4} = 6.0$, $J_{3,5} = 1.5$, $J_{4,5} = 3.5$, $J_{1,9} = 6.7$, $J_{10_A,10_B} = 15.0$.

* Line at higher field overlapped by HDO (δ 4.70) signal.

† Overlapped.

‡ Partly overlapped.

§ *s (br)* with fine structure.

Table 2. ^{13}C NMR data for compounds **1** and **2** [20 MHz, D_2O, ppm from TMS; dioxane (67.4 ppm) as int. standard]

		2	
C	**1** [17]	α	β
1	96.29	92.20	95.31
3	140.43	140.16	141.31
4	106.06	105.28	105.28
5	43.26	43.75	45.12
6	81.36	81.45	82.04
7	129.41	130.96	129.69
8	147.57	147.38	147.38
9	47.18	47.19	48.73
10	60.28	60.75	60.75

Table 3. Antimicrobial activity

	Concentration of **2** (mg/ml)					
Microorganism	16	8	4	2	1	0.5
Staphylococcus aureus ATCC 6538P					+	
Micrococcus luteus ATCC 9341			+			
Bacillus subtilis ATCC 6633						+
B. cereus ATCC 11778						+
Escherichia coli ATCC 10536		+				
Candida albicans ATCC 10231	−					
Saccharomyces cerevisiae ATCC 9763	±					
Pseudomonas aeruginosa ATCC 9027			+			
Aspergillus niger ATCC 16404					+	
Penicillium crysogenum ISS 1		+				

Inhibition zone: +, > 14 mm; ±, < 14 mm; −, 0 mm.

ATCC, American Type Culture Collection; ISS, Istituto Superiore Sanità Collection.

The importance of this simple and highly efficient method for obtaining 2 is apparent when one considers the potential use of iridoid glucosides and aglycones as chiral precursors for the synthesis of natural products with cyclopentanoid skeletons.

Although our initial target was to obtain 2 in the highest yields, we have tested the same method on two other iridoid glucosides (unedoside 3 [20, 21] and macfadyenoside 4 [22]) with different substitution patterns and polarities. The results (see Experimental) were very satisfactory as the corresponding aglycones were extracted with yields comparable (~ 78–85%) to those of 2.

EXPERIMENTAL

CC: 'washed' silica gel (Merck Si gel 70–230 mesh treated overnight with 2M HCl, washed with hot H_2O until free of Cl^- ions, dried and activated at 120° for 24 hr); TLC: silica gel 60 F_{254} Merck plates. Spray reagents: 1 M H_2SO_4, heated at 120° (TLC); vanillin (vanillin 1 g, 2 ml conc HCl, 100 ml MeOH) heated at 100° (paper test).

General procedure for extraction of aucubigenin (2). Aucubin (1) (1 g, obtained from *Aucuba japonica* [7]) was dissolved in 20 ml H_2O containing β-glucosidase (300 mg, Fluka EC 3.2.1.21) and left at 36° until 1 was completely hydrolysed (*ca* 2 hr). The reaction was monitored by TLC (EtOAc–MeOH 4:1). The green soln, was then transferred on a liquid–liquid extractor and extracted with EtOAc (*ca* 6 hr) until it gave a negative vanillin reaction (paper test). The EtOAc soln was dried (Na_2SO_4) and the solvent evaporated *in vacuo*. The amorphous residue, checked on TLC (EtOAc–MeOH, 9:1), contained chromatographically pure 2 (510 mg, yield 96%).

For analytical purposes, part of this residue was chromatographed on 'washed' silica gel with EtOAc–MeOH (9:1). Crystalline 2 separated spontaneously from the column fractions. It was filtered and re-crystallized from EtOAc (mp 110°).

Antimicrobial tests. Bacteria were grown on brain heart infusion broth (BHI Difco) at 32° for 18–24 hr, yeasts on sabouraud dextrose broth (Difco) at 22° for 24 hr and moulds on sabouraud dextrose agar (Difco) at 22° for 1 week. The media were inoculated with one loopful of growth from a slant. At the end of incubation period the broth cultures were standardized to reduce the microbial count to 10^6 cfu/ml. The mould agar was washed using saline peptone water (Difco) containing 0.05% polysorbate 80 and then diluted to reduce the spore count to about 10^7 spores/ml. Standard II Nutrient Agar (Merck) containing KH_2PO_4, pH 7.2 (bacteria) and pH 5.5 (yeasts and moulds), was poured into flat-bottomed Petri dishes and allowed to solidify, for not more than 1 hr, at room temp. 0.1 ml of inoculum was spread on the dried surface by streaking a swab over the entire agar surface. Paper discs (Whatman AA ϕ 13 mm) which contained serial dilutions (H_2O) of aucubigenin (2) (0.1 ml) were then placed on the agar and the plates incubated at 32° (18–24 hr) for bacteria and at 22° for yeasts and moulds (18 hr and 7 days, respectively).

Extraction of aglycones of 3 *and* 4. The same procedure as that used for 2 gave the crude aglycones of unedoside (3) and macfadyenoside (4) in good yields (78 and 85%, respectively). The ^1H NMR spectra (300 MHz) of these aglycones were as follows: *Aglycone of* 3 (acetone-d_6): (β-form), δ 1.9–2.1 (*m*, covered by solvent signals, H-5), 2.27 (1H, *dd*, $J_{1,9}$ = 9.0 Hz, $J_{5,9}$ = 7.0 Hz, H-9), 3.46 (1H, *d*, $J_{7,8}$ = 3.0 Hz, H-8), 3.66 (1H, *dd*, $J_{6,7}$ = 1.4 Hz, $J_{7,8}$ = 3.0 Hz, H-7), 3.85 (1H, *d* (*br*), $J_{5,6}$ = 6.0 Hz, $J_{6,7}$ = 1.4 Hz, H-6), 4.59 (1H, *d*, $J_{1,9}$ = 9.0 Hz, H-1), 4.95 (1H, *pt*, $J_{3,4}$ = 6.0 Hz, $J_{4,5}$ = 5.4 Hz, H-4), 6.26 (1H, *dd*, $J_{3,4}$ = 6.0 Hz, $J_{3,5}$ = 1.9 Hz, H-3); *Aglycone of* 4 (D_2O): (α-form), δ 2.68 (1H, *d*, $J_{1,9}$ = 3.9 Hz, H-9), 3.58 (1H, *d*, $J_{6,7}$ = 1.0 Hz, H-7), 3.80 centre AX (2H, *dd*, 2H-10, J_{AX} = 15.0 Hz), 4.18 (1H, *d*, $J_{6,7}$ = 1.0 Hz, H-6), 4.95 (1H, *d*, $J_{3,4}$ = 6.3 Hz, H-4), 5.49 (1H, *d*, $J_{1,9}$ = 3.9 Hz, H-1), 6.25 (1H, *d*, $J_{3,4}$ = 6.3 Hz, H-3).

Acknowledgement—This work was supported by the National Research Council of Italy (CNR) under the 'Progetto Finalizzato del CNR Chimica Fine e Secondaria'.

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