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AN IRIDOID DIESTER FROM *VALERIANA OFFICINALIS* VAR. *SAMBUCIFOLIA* HAIRY ROOTS

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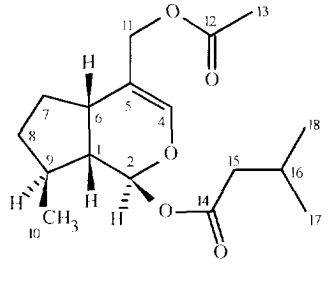
Key Word Index—*Valeriana officinalis*; Valerianaceae; hairy roots; iridoid; valepotriate; valdiate.

Abstract—A new iridoid diester (1*R*,2*S*,6*S*,9*S*)-5-acetyloxymethyl-9-methyl-3-oxabicyclo[4.3.0.]non-4-en-2-yl isovalerate), which we have named valdiate, was isolated from the hairy roots of *Valeriana officinalis* var. *sambucifolia* Mikan transformed with *Agrobacterium rhizogenes* R1601. It was characterized by its chemical and physical data. Together with valdiate, five valepotriates were isolated and identified by means of mass spectrometry and ^{1}H and ^{13}C NMR spectrometry. The analysis of the non-transformed plant roots belonging to the same species, indicated the absence of valdiate and the presence of four valepotriates only.

INTRODUCTION

In our current investigation of the secondary metabolites produced by the transformed roots of the Valerianaceae [1], we focused our attention on the esterified iridoid content of *Valeriana officinalis* L. var. *sambucifolia* Mikan. In this report, we describe the isolation and the structural elucidation of a new iridoid diester, which we have called valdiate (**1**) [(1*R*,2*S*,6*S*,9*S*)-5-acetyloxymethyl-9-methyl-3-oxabicyclo[4.3.0.]non-4-en-2-yl isovalerate)], (alternative name: 11-acetoxy-1-isovaleroyl-iridodial). Together with **1**, five valepotriates, 7-desisovaleroyl-7-acetylvaltrate, didrovaltrate, isovaleroxyhydroxydidrovaltrate, isovaltrate and valtrate, were isolated and characterized. In order to evaluate the effects of *Agrobacterium*-mediated transformation on the secondary metabolism, non-transformed roots of *V. officinalis* var. *sambucifolia* were also examined by the same procedure.



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RESULTS AND DISCUSSION

Extraction of the dried hairy roots from *V. officinalis* var. *sambucifolia* by dichloromethane yielded a crude extract which was fractionated by liquid chromatography on Lichroprep RP-18 column. Pure valdiate (**1**) was obtained by semi-preparative HPLC on reversed-phase silica.

Compound **1**, molecular formula $C_{17}H_{26}O_5$, was readily identified by standard means (MS, ^{1}H NMR, IR) as a diester. The IR spectrum (neat on KBr) showed in addition to two dominant ester bands at 1760 and 1735 cm^{-1}, a strong and sharp absorption at 1670 cm^{-1} suggesting the presence of an enol ether function. The mass spectral fragmentation pattern was indicative of an acetate ($[M-59]^+$), as well as of an isovalerate group ($[M-101]^+$). The elemental composition of the corresponding fragment peaks was ascertained by high resolution mass spectrometry. These findings combined with the ^{1}H and ^{13}C NMR spectral data strongly suggested **1** to have a C_{10} iridoid skeleton.

A ^{13}C resonance at δ91.5 (i.e. C-2) together with the enol ether absorption seen in the IR spectrum, was indicative of the bicyclic lactol skeleton. The *cis*-connection of the two rings was deduced from a 7.2 Hz scalar coupling between the bridgehead protons. A homonuclear coupling of 4.8 Hz between the hydrogen atoms H-1 and H-2 is similar to that of valepotriates found in *Valeriana officinalis* s.l. [2]. It suggested that these atoms are *trans* to each other, and consequently, the lactol ester function must be *cis* related to the adjacent bridgehead hydrogen H-1. Further structural details, in particular the presence of the *exo*-cyclic allylic methylene unit (H_2 C-11)

and the positioning of the methyl group at the five-membered ring, were ascertained by a shift correlation experiment (H,H-COSY [3]). The point of attachment of the methyl group at C-9 is in full agreement with a normal isoprenoid ramification pattern. More subtle stereochemical questions were answered by a 2D ROESY experiment [4, 5]. It showed a strong cross peak between H-2 and H-9 indicative of a strong dipolar coupling between these atoms. This requires the methyl group at C-9 to be *syn* oriented with respect to the adjacent bridgehead hydrogen atom H-1.

The most difficult part of the structure determination consisted in assigning the site of attachment of the acetate and of the isovalerate function. In order to answer this question, we examined heteronuclear long range couplings. Use was made of a HMQC pulse sequence [6] with a J-filter [7] to suppress one bond coupling. The 2D experiment was optimized for the observation of 7 Hz $^1H/^{13}C$ couplings. In this study, the carboxyl resonance at higher field (δ170.9) was readily recognized as belonging to the acetate since it correlated with the acetyl protons. Moreover, it was found to correlate with the allylic methylene protons (H_2C-11). In a complementary fashion, the carboxyl resonance at lower field (δ171.9) showed a cross peak with the methylene group (H_2C-15) of isovalerate, and it correlated with the lactol proton (HC-2).

The spectroscopic data of **1**, except for differences in assignment of ^{13}C resonances, were almost identical to those reported for teucrein [8]. This diacetate of the lactol of iridodial was isolated from *Teucrium marum* L. (Labiatae) [8]. Like **1**, it was levorotatory $[\alpha]_D^{20}$ −73.5° (C_6H_6; *c* 0.3), and obviously has the same iridoidal skeleton as valdiate (**1**). Teucrein has been chemically related to (*S*)-citronellal. We can, therefore, safely conclude that teucrein and **1** have the same absolute configuration at C-9.

The valepotriates isolated together with **1** were identified as 7-desisovaleroyl-7-acetylvaltrate (20 mg), didrovaltrate (10 mg), isovaleroxyhydroxydidrovaltrate (55 mg), isovaltrate (120 mg) and valtrate (95 mg), by means of mass spectrometry and a comparison of their ^1H and ^{13}C NMR data with published data [2, 9–13]. By comparison, a dichloromethane extract of the roots of nine-month-old non-transformed plants grown in the field was fractionated by liquid chromatography as described below. Didrovaltrate (10 mg), isovaleroxyhydroxydidrovaltrate (32 mg), isovaltrate (130 mg) and valtrate (30 mg) were isolated and identified by means of EI-mass and NMR spectroscopy. However, valdiate and 7-desisovaleroyl-7-acetylvaltrate, isolated from the hairy roots, were not detected in the non-transformed roots. The *Agrobacterium*-mediated transformation of *Valeriana officinalis* var. *sambucifolia* was associated with a four-fold increase in valepotriate content [1] as well as with the biosynthesis of secondary metabolites not present in the roots of the non-transformed plants. These major quantitative and qualitative changes may undoubtedly be beneficial for obtaining esterified iridoids by tissue culture.

EXPERIMENTAL

Bacterial strain. Agrobacterium rhizogenes strain R1601 [14] was grown on solid YMB medium [15] supplemented with 100 mg l^{-1} kanamycin and subcultured at 1 month interval.

Plant material. The seeds of *Valeriana officinalis* var. *sambucifolia* (Station fédérale de recherches agronomiques, centre d'arboriculture et d'horticulture des Fougères, Conthey, Switzerland) were surface sterilized (15 min) in 2% NaClO soln, washed (3 × 20 min) with sterile water and left to germinate. The plantlets were transferred to solid Murashige and Skoog medium [16] supplemented with 2% sucrose (MS-2) and grown for 3 weeks at 25° in the light. Plants originating from the same batch of seeds and grown in the field were identified by comparison with the authentic herbarium specimens of the Conservatoire et Jardin Botaniques in Geneva.

Transformation with A. rhizogenes. The plantlets were wounded and infected with *A. rhizogenes*. The hairy roots were excised and cleared of bacteria on MS-2 solid medium containing 0.25 g l^{-1} cefotaxime and 1 g l^{-1} ampicillin. The sterile hairy roots were then cultured in hormone-free half strength Gamborg B5 liquid medium [17] supplemented with 2% sucrose on a gyratory shaker in the dark.

Opine analysis. To prove the transformation the opines were extracted and identified by paper electrophoresis by comparison with authentic samples [18].

Extraction. Lyophilized roots (100 g, either hairy roots or non-transformed roots) were extracted with stirring at room temp, with 3 × 700 ml CH_2Cl_2 for 1 hr. The CH_2Cl_2 extract was concd to 600 ml under red. pres. at 25°, neutralized with 2 × 250 ml $NaHCO_3$ (3%), washed with NaCl (5%) and dried with dry Na_2SO_4. The extract was further concd to 200 ml, neutralized again with NaOH (0.1%), washed with NaCl (5%) and dried with dry Na_2SO_4 [19]. CH_2Cl_2 was evapd under red. pres. at 25°. The residue was dissolved in MeOH and filtered. MeOH was evapd under red. pres. at 25°. The amount of crude extract was 7.4 g for the hairy roots and 2.1 g for the non-transformed roots.

Isolation of valdiate the valepotriates. Crude extract (0.5 g) was chromatographed on two Lobar Lichroprep RP-18 columns joined in series, using MeOH–H_2O (7:3) at a flow rate of 1 ml min^{-1}. The effluent was monitored by TLC [silica gel 60 F_{254} with CH_2Cl_2–*n*-PrOH–Me_2CO, (198:1:1)]. Similar frs were combined and subjected to semi-prep. HPLC on a Varioprep® Nucleosil C-18 column (25 × 2.1 cm) fitted with a guard column (5 × 2.1 cm), using MeOH–H_2O (7:3) at a flow rate of 10 ml min^{-1} and detection at 208 nm.

NMR spectral data for valdiate (**1**). ^1H NMR ($CDCl_3$, 400 MHz); δ0.98 (6H, 2*d*, *J* = 6.5 Hz, H-17 and H-18), 1.10 (3H, *d*, *J* = 6.2 Hz, H-10), 1.21 (1H, *m*, H-8), 1.52 (1H, *m*, H-7), 1.79 (1H, *td*, *J* = 7.2, 4.8 Hz, H-1), 1.85–2.15 (4H, *m*, H-9, H'-8, H'-7, H-16), 2.06 (3H, *s*, OAc), 2.25 (2H, *m*, H-15), 2.69 (1H, *br q*, *J* = 7.2 Hz, H-6), 4.40 and 4.59 (2H, [AB], *J* = 12.2 Hz, H-11), 5.91 (1H, *d*, *J* = 4.8 Hz, H-2), 6.34 (1H,

br s, H-4); ^{13}C NMR ($CDCl_3$, 100 MHz); δ20.15 (C-10), 21.0 (C-13), 22.31 (C-17 and C-18), 25.63 (C-16), 30.01 (C-7), 33.0 (C-8), 34.97 and 35.04 (C-9 and C-6), 43.91 (C-15), 47.97 (C-1), 64.03 (C-11), 91.46 (C-2), 113.0 (C-5), 140.2 (C-4), 170.9 (C-12), 171.9 (C-14).

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