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Research Article

Valerian-Derived Sedative Agents. I. On the Structure and Spectral Assignment of the Constituents of Valmane Using the Selective INEPT Nuclear Magnetic Resonance Technique

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The valepotriates, a group of chemically unstable iridoid triesters possessing sedative activity, contain various ester groups at the C-1, C-7, and C-11 positions. Using the selective INEPT NMR technique and employing a suitable polarization delay for long-range coupling, it was possible to achieve the assignment and location of the ester groups directly, without ambiguity, and without chemical modification. Six valepotriates isolated from Valmane tablets served as examples to demonstrate the utility of this NMR technique. During the course of this work, the "acevaltrate" fraction was shown to be a mixture of 1-α-acevaltrate (3) and 7-β-acevaltrate (4), the structures of valtrate (1) and didrovaltrate (2) were confirmed directly, and two new valepotriates, 5a and 5b, were obtained as an inseparable mixture and characterized.

KEY WORDS: valerian; Valmane; valepotriates; isolation; structure elucidation; spectroscopic assignment; selective INEPT NMR technique.

INTRODUCTION

The product Valmane, which is available in many parts of Europe, is one of more than 80 products containing valerian preparations which are widely recommended for use as mild sedative agents (1–8). Valmane is reported (3,9,10) to contain three valepotriates (valtrate, didrovaltrate, and acevaltrate) which are claimed to be responsible for the action of this pharmaceutical preparation.

The valepotriates are chemically unstable iridoid triesters in which the various hydroxy groups are esterified with acetic, isovaleric, hydroxyisovaleric, β-methyl valeric, and related acids (3,11). Evidence to assign these structures has previously been based on interpretation of their carbon-13 NMR chemical shift correlation data (11) and mass spectral fragmentation schemes (12), as well as evidence from chemical degradation reactions, and has resulted in the revision of some earlier proposed structures (13). In order to assign the structures of some new isolates of Valmane tablets, and also to establish a method for the direct and unambiguous assignment of the location of the ester groups, we chose to reexamine the accepted structures of valtrate (1) and didrovaltrate (2), and to establish the nature of the "acevaltrate" fraction, before proceeding to the structures of the new isolates.

The technique that we chose to examine the placement of the ester groups is the selective INEPT NMR technique (14). This technique relies on the observation of the different two-, three-, and four-bond couplings between protons and carbons, and we have found it to be particularly useful both for the unambiguous assignment of carbon-13 NMR spectra and for the structure elucidation of natural products, even when only limited quantities are available (15,16).

MATERIALS AND METHODS

Valmane tablets (Lyssia GmbH, Wiesbaden, Germany, formerly distributed by Kali-Chemie Pharma GmbH, Hannover, Germany), purchased locally in pharmacies in Oberammergau (Lyssia Lot CH.-B 8431) and Munich (Kali-Chemie Lot 150684), Germany, were ground to a powder and extracted exhaustively with chloroform ($CHCl_3$) at room temperature. The pooled $CHCl_3$ extracts were filtered and concentrated *in vacuo* below 35°C to yield a yellowish resin. This resin was subjected to column chromatography on a silica gel (70 to 230 mesh, 60 Å) column. Elution was initiated with petroleum ether and continued with eluants of increasing polarity by adding various percentages of $CHCl_3$. The column fractions which contained valtrate (1) (eluted with 2–5% $CHCl_3$ in petroleum ether; weight percentage, 16.3%), didrovaltrate (2) (5–10% $CHCl_3$ in petroleum ether; 81.6%), isolates 3 and 4 (10–20% $CHCl_3$ in petroleum ether;

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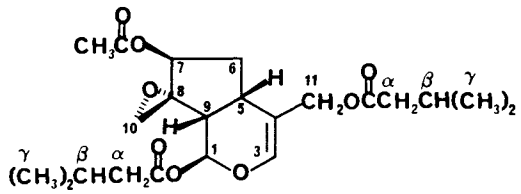
0.9 and 0.1%, respectively), and isolates **5a** and **5b** (20% $CHCl_3$ in petroleum ether; 1.1%) were combined separately and purified by preparative thin-layer chromatography (TLC) using the following solvent systems: petroleum ether–ethyl acetate–acetone (100:8:8, v/v), $CHCl_3$–ethyl acetate–acetone (100:2:2, v/v), and toluene–ethyl acetate (3:1, v/v) (17) (Fig. 1).

Proton NMR spectra were obtained on either a Varian XL-300 NMR spectrometer operating at 299.9 MHz or a Nicolet NT-360 instrument operating at 361.1 MHz. ^{13}C-NMR spectra were obtained on a Nicolet NT-360 NMR spectrometer operating at 90.8 MHz. All chemical shifts are reported as parts per million. Samples were dissolved in deuterochloroform for NMR analysis, and tetramethylsilane (δ 0 ppm) was used as the internal standard. An APT experiment was used for the determination of the multiplicity of the carbon atoms. The selective INEPT NMR experiments (14) were performed on a Nicolet NT-360 spectrometer. Data sets of 16K covering a spectral width of 10,000 Hz were acquired. Proton pulse widths were calibrated by using a sample of AcOH in 10% C_6D_6 (^{n}J = 6.7 Hz) in a 5-mm tube. The radiofrequency field strength for the soft proton pulse was of the order of 25 Hz in these experiments. For H-1, H-7, and H-11, a value of 3 Hz was used for $^{3}J_{CH}$ and for acetate protons a value of 7 Hz was used for $^{2}J_{CH}$. Different values were used for other protons depending on the two-bond or three-bond couplings to be emphasized. One thousand acquisitions for 1 and 2, 10,000 for 3 and **5a/5b**, and 40,000 for 4 were accumulated in each irradiation experiment. Mass spectra were measured by the direct-inlet method on a Finnigan MAT-90 mass spectrometer operating in the positive CI mode using methane as the ionizing gas. The FAB mass spectra obtained for **5a/5b** were measured on the same mass spectrometer in the positive and negative ion modes using glycerol as the matrix.

RESULTS

Valtrate (1) exhibited the following spectral data: ^{1}H-NMR (299.9 MHz, $CDCl_3$) δ 6.66 (1H, s, H-3), 5.95 (1H, d, J = 10.1, H-1), 5.83 (1H, dd, J = 2.8, 2.8, H-6), 5.33 (1H, d, J = 2.9, H-7), 4.70 (1H, d, J = 12.3, H-11), 4.63 (1H, d, J = 12.3, H-11), 3.40 (1H, dd, J = 10.0, 2.6, H-9), 2.99 (1H, d, J = 4.9, H-10), 2.87 (1H, d, J = 4.9, H-10), 2.02 (3H, s, OAc), 0.96 (3H, s, isovalerate CH_3), 0.94 (3H, s, isovalerate CH_3), 0.92 (3H, s, isovalerate CH_3), and 0.90 (3H, s, isovalerate CH_3); ^{13}C-NMR (90.8 MHz, $CDCl_3$) δ 172.31 (s, CO at C-7), 170.72 (s, CO at C-11), 170.19 (s, CO at C-1), 148.35 (d, C-3), 140.77 (s, C-5), 118.51 (d, C-6), 108.17 (s, C-4), 92.42 (d, C-1), 82.90 (d, C-7), 64.05 (s, C-8), 60.73 (t, C-11), 47.78 (t, C-10), 43.24 (t, isovalerate CH_2), 42.91 (t, isovalerate CH_2), 42.85 (d, C-9), 25.72 (d, isovalerate CH), 25.51 (d, isovalerate CH), 22.24 (q, isovalerate CH_3), 22.13 (q, isovalerate CH_3), 20.88 (q, acetate CH_3); MS (positive CI) *m/z* (rel. int.) 423 (MH^+, 0.4%), 364 (4), 363 (19), 322 (4), 321 (22), 291 (2), 279 (1), 262 (5), 261 (21), 220 (14), 219 (100), 191 (24), 177 (20), 103 (14).

Didrovaltrate (2) exhibited the following spectral data: ^{1}H-NMR (299.9 MHz, $CDCl_3$) δ 6.49 (1H, s, H-3), 5.81 (1H, d, J = 5.4, H-1), 4.92 (1H, dd, J = 5.7, 5.7, H-7), 4.66 (1H, d, J = 12.2, H-11), 4.43 (1H, d, J = 12.2, H-11), 3.03 (1H, d, J = 5.0, H-10), 2.94 (1H, m, H-5), 2.79 (1H, d, J = 5.0,



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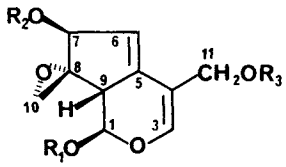

	R_1	R_2	R_3
1	isovaleryl	isovaleryl	acetyl
3	α-acetoxyisovaleryl	isovaleryl	acetyl
4	isovaleryl	β-acetoxyisovaleryl	acetyl
5a	isovaleryl	isovaleryl	β-hydroxyisovaleryl
5b	isovaleryl	β-methylvaleryl	β-hydroxyisovaleryl

Fig. 1. Structures of the valepotriates isolated from Valmane tablets.

H-10), 2.69 (1H, dd, J = 8.4, 5.4, H-9), 2.02 (3H, s, OAc), 0.94 (6H, s, 2 X isovalerate CH_3), and 0.92 (6H, s, 2 X isovalerate CH_3); ^{13}C-NMR (90.8 MHz, $CDCl_3$) δ 172.75 (s, CO at C-11), 170.90 (s, CO at C-1), 169.61 (s, CO at C-7), 142.01 (d, C-3), 110.57 (s, C-4), 88.04 (d, C-1), 76.41 (d, C-7), 63.88 (s, C-8), 63.07 (t, C-11), 48.59 (t, C-10), 43.15 (t, isovalerate CH_2), 42.96 (t, isovalerate CH_2), 39.15 (d, C-9), 34.77 (t, C-6), 32.21 (d, C-5), 25.51 (d, isovalerate CH), 25.34 (d, isovalerate CH), 22.19 (q, isovalerate CH_3), 22.12 (q, isovalerate CH_3), 20.78 (q, acetate CH_3); MS (positive CI) m/z (rel. int.) 425 (MH^+, 5%), 397 (4), 323 (100), 263 (4), 257 (4), 239 (2), 221 (9), 179 (20), 161 (43), 151 (4), 133 (18), 119 (11), 103 (6).

Valmane isolate **3** exhibited the following spectral data: ^{1}H-NMR (299.9 MHz, $CDCl_3$) δ 6.67 (1H, s, H-3), 6.00 (1H, d, J = 10.3, H-1), 5.86 (1H, dd, J = 2.7, 2.7, H-6), 5.35 (1H, d, J = 2.6, H-7), 4.93 [1H, d, J = 4.2, acetoxy isovalerate CH(α)], 4.71 (1H, d, J = 12.2, H-11), 4.63 (1H, d, J = 12.3, H-11), 3.45 (1H, dd, J = 10.1, 2.5, H-9), 2.99 (1H, d, J = 4.8, H-10), 2.87 (1H, d, J = 4.8, H-10), 2.15 (3H, s, α-O*Ac* isovalerate CH_3 at C-1), 2.03 (3H, s, OAc at C-11), 1.02 (3H, d, J = 6.9, acetoxy *isovalerate* CH_3), 0.98 (3H, d, J = 6.8, acetoxy *isovalerate* CH_3), 0.93 (3H, s, isovalerate CH_3), 0.91 (3H, s, isovalerate CH_3); ^{13}C-NMR (90.8 MHz, $CDCl_3$) δ 172.42 (s, CO at C-7), 170.83 (s, CO at C-11), 170.51 (s, α-acetoxy CO at C-1), 167.44 (s, CO at C-1), 148.15 (d, C-3), 140.51 (s, C-5), 119.04 (d, C-6), 108.24 (s, C-4), 93.20 (d, C-1), 82.98 (d, C-7), 76.01 (d, acetoxy isovalerate CH(α)), 64.06 (s, C-8), 60.77 (t, C-11), 47.80 (t, C-10), 43.34 (t, isovalerate CH_2), 42.91 (d, C-9), 29.99 [d, acetoxy isovalera te CH(β)], 25.83 (d, isovalerate CH), 22.33 (q, isovalerate CH_3), 20.98 (q, acetate CH_3), 20.56 (q, acetate CH_3), 18.85 (q, acetoxy isovalerate CH_3), 16.54 (q, acetoxy isovalerate CH_3); MS (positive CI) m/z (rel. int.) 481 (MH^+, 0.3%), 423 (9), 422 (6), 421 (25), 380 (4), 379 (19), 361 (1), 323 (2), 322 (1), 321 (7), 319 (2), 261 (5), 221 (3), 220 (14), 219 (100), 203 (2), 191 (14), 177 (4), 161 (8), 159 (4), 149 (2), 143 (56), 133 (3), 103 (16).

Valmane isolate **4** exhibited the following spectral data: ^{1}H-NMR (299.9 MHz, $CDCl_3$) δ 6.68 (1H, s, H-3), 5.96 (1H, d, J = 10.2, H-1), 5.83 (1H, dd, J = 2.8, 2.8, H-6), 5.35 (1H, d, J = 2.9, H-7), 4.72 (1H, d, J = 12.9, H-11), 4.63 (1H, d, J = 12.2, H-11), 3.39 (1H, dd, J = 10.3, 2.6, H-9), 3.00 (1H, d, J = 4.8, H-10), 2.90 (1H, d, J = 12.4, acetoxy isovalerate CH_2), 2.88 (1H, d, J = 4.8, H-10), 2.84 (1H, d, J = 12.4, acetoxy isovalerate CH_2), 2.04 (3H, s, OAc at C-11), 1.95 (3H, s, β-O*Ac* isovalerate CH_3 at C-7), 1.49 (3H, s, acetoxy *isovalerate* CH_3), 1.48 (3H, s, acetoxy *isovalerate* CH_3), 0.97 (3H, s, isovalerate CH_3), 0.95 (3H, s, isovalerate CH_3); ^{13}C-NMR (90.8 MHz, $CDCl_3$) δ 170.85 (s, CO at C-11), 170.35 (s, β-acetoxy CO at C-7), 170.30 (s, CO at C-1), 169.49 (s, CO at C-7), 148.63 (d, C-3), 141.10 (s, C-5), 118.31 (d, C-6), 108.21 (s, C-4), 92.47 (d, C-1), 83.29 (d, C-7), 79.22 [s, β-acetoxy isovalerate C(β)], 64.08 (s, C-8), 60.77 (t, C-11), 47.87 (t, C-10), 44.02 [t, β-acetoxy isovalerate CH_2(α)], 43.01 (t, isovalerate CH_2), 42.96 (d, C-9), 26.74 (q, acetoxy *isovalerate* CH_3), 26.66 (q, acetoxy *isovalerate* CH_3), 25.62 (d, isovalerate CH), 22.36 (q, isovalerate CH_3), 22.24 (q, β-acetoxy *isovalerate* CH_3 at C-7), 20.99 (q, acetate CH_3 at C-11); MS (positive CI) m/z (rel. int.) 481 (MH^+, 0.3%), 423 (1), 381 (5), 362 (10), 361 (52), 353 (5), 323 (25), 321 (20), 281 (8), 279 (5), 277 (24), 261 (7), 231 (7), 221 (11), 220 (15), 219 (100), 203 (7), 193 (4), 191 (35), 177 (35), 161 (45), 159 (9), 149 (21), 143 (14), 133 (26), 103 (61).

Valmane isolate **5a** and **5b** exhibited the following spectral data: ^{1}H-NMR (361.1 MHz, $CDCl_3$) δ 6.69 (s, H-3), 5.95 (d, J = 10.1, H-1), 5.84 (dd, J = 2.7, 2.7, H-6), 5.34 (d, J = 2.9, H-7), 4.78 (d, J = 12.3, H-11), 4.68 (d, J = 12.3, H-11), 3.40 (dd, J = 10.2, 2.4, H-9), 3.00 (d, J = 4.9, H-10), 2.88 (d, J = 4.8, H-10), 2.47 (s, β-hydroxy isovalerate CH_2), 1.84 (m, β-methyl valerate CH), 1.25 (s, β-hydroxy isovalerate CH_3), 0.97 (s, isovalerate CH_3), 0.95 (s, isovalerate CH_3), 0.93 (s, isovalerate CH_3), 0.89 (d, J = 6.6, β-methyl valerate CH_3), 0.86 (d, J = 7.1, β-methyl valerate CH_3); ^{13}C-NMR (90.8 MHz, $CDCl_3$)δ 172.68 (s, CO at C-11 and **5b** β-methyl valerate CO at C-7), 172.43 (s, **5a** isovalerate CO at C-7), 170.31 (s, CO at C-1), 148.72 (d, C-3), 140.71 (s, C-5), 118.69 (d, C-6), 107.95 (s, C-4), 92.53 (d, C-1), 82.93 (d, C-7), 69.03 [s, β-hydroxy isovalerate C(β)], 64.10 (s, C-8), 60.88 (t, C-11), 47.86 (t, C-10), 43.31 (t, **5a** isovalerate CH_2 at C-7), 42.98 (t, isovalerate CH_2 at C-1), 42.92 (d, C-9), 41.36 [t, **5b** β-methyl valerate CH_2(α) at C-7], 31.94 [d, **5b** β-methyl valerate CH(β) at C-7], 29.27 [t, **5b** β-methyl valerate CH_2(γ) at C-7], 29.16 (q, β-hydroxy isovalerate CH_3), 25.76 (d, **5a** isovalerate CH at C-7), 25.59 (d, isovalerate CH at C-1), 22.32 (q, isovalerate CH_3), 22.21 (q, isovalerate CH_3), 19.22 (q, **5b** β-methyl CH_3 of β-methyl valerate at C-7), 11.28 (q, **5b** terminal CH_3 of β-methyl valerate at C-7); MS (positive CI) m/z (rel. int.) 463 (4%), 439 (4), 435 (2), 421 (4), 394 (3), 393 (13), 380 (8), 379 (39), 378 (9), 377 (40), 364 (5), 363 (23), 340 (3), 339 (16), 323 (6), 294 (4), 293 (20), 280 (3), 279 (10), 278 (16), 277 (100), 275 (7), 261 (13), 249 (15), 233 (5), 177 (23), 159 (7); FAB-MS (positive) m/z (rel. int.) 261 (19%), 203 (18), 193 (27), 191 (26), 177 (100), 161 (51), 159 (48), 149 (58), 119 (35), 117 (10), 103 (31). FAB-MS (negative) m/z (rel. int.) 325 (5%), 311 (5), 293 (3), 279 (3), 201 (6), 191 (6), 189 (4), 175 (6), 117 (100), 115 (62), 101 (84).

DISCUSSION

Valerian preparations are extremely popular mild sedatives used in many European countries, particularly Germany. However, in spite of extensive studies (6,7,18–20), many questions remain about the structures and biological activities of the iridoid triesters, the valepotriates (12,13,21–24), which are purported to be responsible for the sedative activity. Due to the lability of the valepotriates, even under relatively mild conditions (25), attempts to assign their structures employing chemical methods have been rather difficult. Popov and Handjieva (12) had reported the structure elucidation of some valepotriates using mass spectrometry. However, the empirical rules developed by these authors are somewhat confusing and would not allow for the unequivocal structure determination of valepotriates with alternative esterifying groups. Thies *et al.* (11) reported the ^{13}C-NMR chemical shifts of a series of valepotriates at 22.63 MHz, showed how the esterifying ester could be inferred, and indicated the dependence of the ^{13}C shifts on the type and position of the ester substituents. However, direct evidence showing the exact location of the various ester groups was not presented, and the possibility of different esterifying groups was not considered. In examining the problem of simply and precisely placing the ester groups, we chose to

employ the selective INEPT NMR technique, which we have used previously for both spectral assignment and structure elucidation (15,16). The application of this NMR technique as reported here provides the first direct solution to the unambiguous assignment and location of the ester groups in this series of compounds. We began an investigation of the application of this technique with valtrate (1) and didrovaltrate (2), which are the major constituents of Valmane, and the compounds whose structures are most firmly established.

NMR and mass spectral data of valtrate (1) confirmed that this compound has one acetoxy and two isovaleryl groups attached to the C-1, C-7, and C-11 positions (11,12). However, the exact location of each ester group could not be ascribed directly from these preliminary data. It was found that application of the selective INEPT technique, using a suitable polarization delay for long-range ^{1}H–^{13}C coupling, was capable of achieving this structure assignment directly. The rationale for these experiments is that separate irradiation of H-1, H-7, and H-11 will specifically enhance the corresponding carbonyl carbon (three-bond ^{1}H–^{13}C couplings) to which the various ester groups are attached, leading to the unambiguous assignment of these carbonyl carbon resonances. Furthermore, irradiation of the acetate methyl group protons will selectively enhance only the carbonyl carbon (two-bond coupling) to which the acetate methyl group is attached. Therefore, by approaching each ester carbonyl group from two bonding directions, the location of these ester groups can be assigned unambiguously.

The selective INEPT experiments carried out for valtrate (1) (Fig. 2) revealed that irradiation of H-1 resulted in the enhancement of the carbonyl signal at 170.19 ppm (Fig. 2b). Irradiation of H-7 selectively enhanced the carbonyl signal at 172.31 ppm (Fig. 2c), and irradiation of H-11 enhanced the carbonyl signal at 170.72 ppm (Fig. 2d). When the methyl of the acetate group was irradiated, using a delay corresponding to J = 7 Hz, enhancement of the carbonyl signal at 170.72 ppm was observed (Fig. 2e). Thus, the acetoxy group could be placed at C-11, leaving the two isovaleryl groups to be located at C-1 and C-7. This conclusion is in agreement with the published structure 1 of valtrate (11).

The CI-MS of didrovaltrate (2) showed that this compound also contained two isovaleryl [*m/z* 221, MH^{+}-2(102)] and one acetoxy (*m/z* 161, 221-60) groups. To determine the location of these ester groups, a series of selective INEPT experiments was performed (Fig. 3). Separate irradiations of H-1 (Fig. 3b), H-7 (Fig. 3c), and H-11 (Figs. 3e and 3f), in the same manner as for 1, resulted in the enhancement of the carbonyl carbons at 170.90, 169.61, and 172.75 ppm, respec-

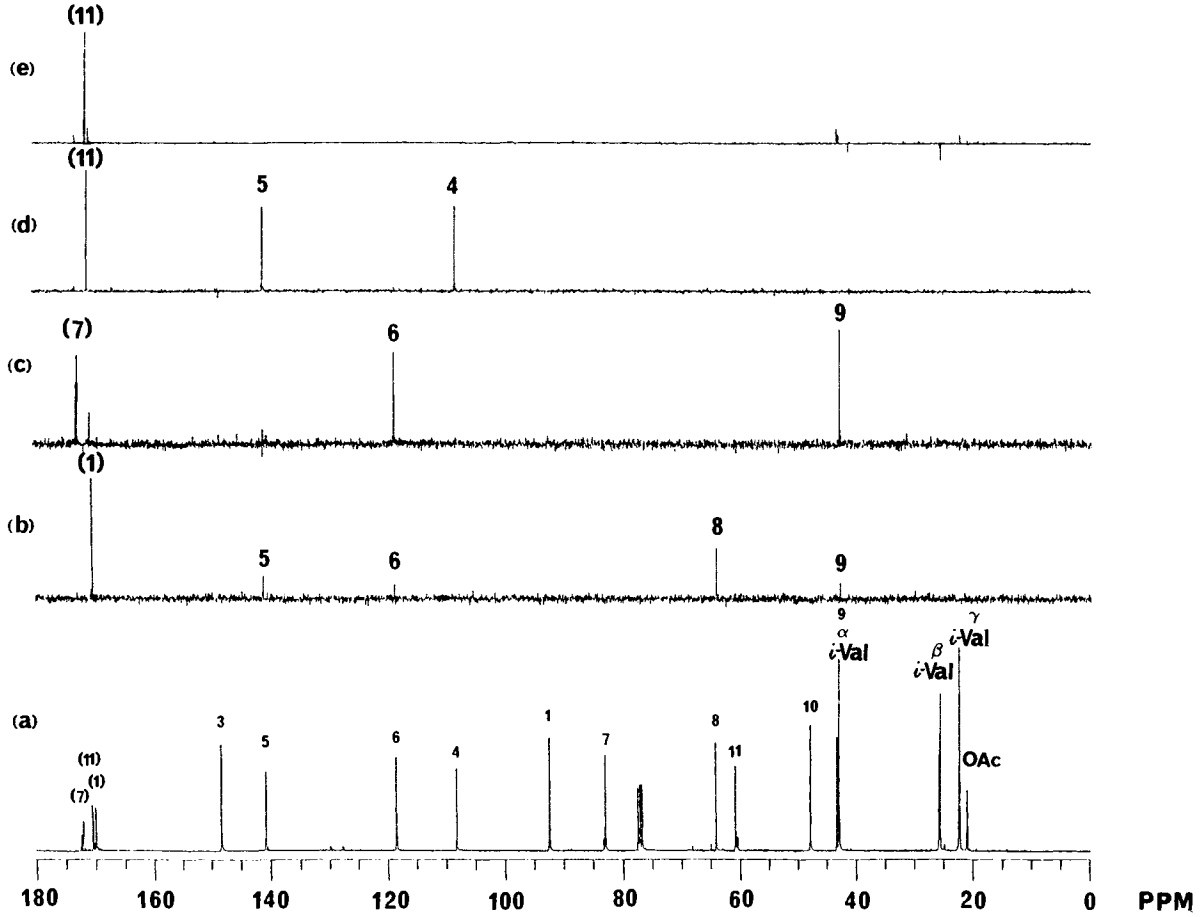

Fig. 2. Selective INEPT experiments of valtrate (1): (a) broad-band decoupled spectrum; (b) irradiation of H-1, J = 3 Hz; (c) irradiation of H-7, J = 3 Hz; (d) irradiation of H-11, J = 3 Hz; (e) irradiation of acetate, J = 7 Hz. The symbols α, β, γ, and δ denote the carbon position of the substituents relative to the carbonyl carbon of the isovalerate/valerate side chains.

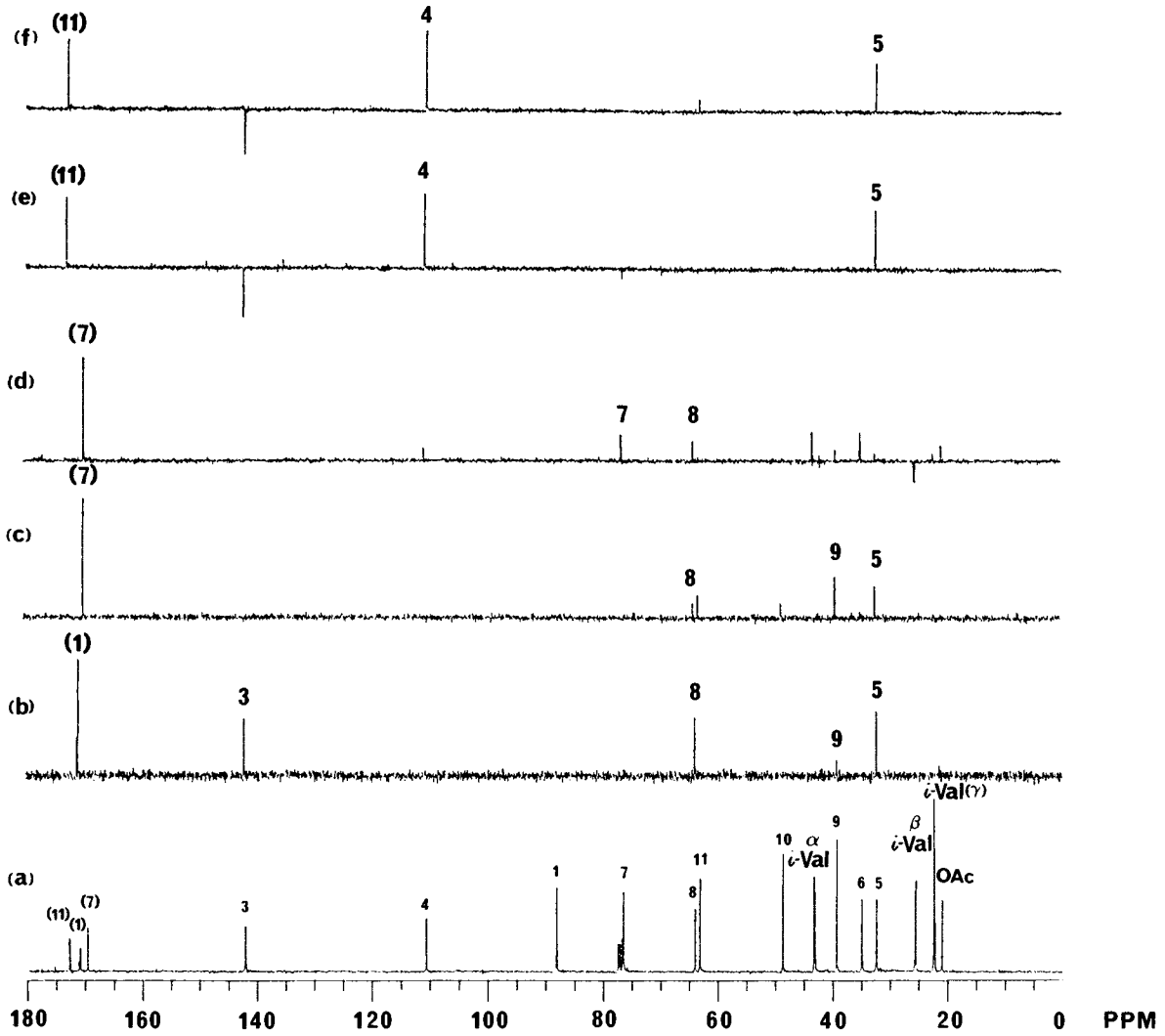

Fig. 3. Selective INEPT experiments of didrovaltrate (2): (a) broad-band decoupled spectrum; (b) irradiation of H-1, J = 3 Hz; (c) irradiation of H-7, J = 3 Hz; (d) irradiation of acetate, J = 7 Hz; (e) irradiation of downfield H-11, J = 3 Hz; (f) irradiation of upfield H-11, J = 3 Hz.

tively. When the acetate methyl group was irradiated, the carbonyl carbon at 169.61 ppm was enhanced (Fig. 3d), indicating that the acetoxy group was at C-7 and the isovaleryl groups were at C-1 and C-11. The structure of didrovaltrate therefore can be unambiguously assigned as 2 (Fig. 1), in agreement with the previously assigned structure (11,13). It is worth reiterating that didrovaltrate (2) is not dihydrovaltrate, but one of its positional isomers.

The presence of three different ester groups in Valmane isolate 3 was evident based on the observation of three prominent fragment ions at m/z 421 (MH^+-60), 379 (MH^+-102), and 321 (MH^+-160). Examination of the NMR data of this isolate further confirmed that these ester groups were acetate, isovalerate, and an acetoxy isovalerate, respectively. Selective INEPT experiments (Fig. 4) showed that separate irradiation of H-1 (Fig. 4b) and the doublet at 4.93 ppm (Fig. 4d) resulted in the enhancement of the same carbonyl carbon at 167.44 ppm, indicating that the acetoxy isovalerate residue was at C-1. Furthermore, separate irradiation of the downfield acetoxy signal at 2.15 ppm (Fig. 4h) and the doublet at 4.93 ppm (Fig. 4e) enhanced the same carbonyl carbon at 170.51 ppm; the acetoxy group could therefore be placed at the α-position of this isovalerate. By irradiating H-11 (Fig. 4f) and the upfield acetoxy signal at 2.03 ppm (Fig. 4g), it was observed that the same carbonyl carbon at 170.83 ppm was enhanced, revealing that the upfield acetoxy group was at C-11. The remaining ester group (isovalerate) was confirmed to be at C-7 by irradiation of H-7, resulting in the enhancement of the carbonyl carbon at 172.42 ppm (Fig. 4c). The structure of Valmane isolate 3 was therefore established to be 1-α-acevaltrate (11).

Investigation of the CI-MS of Valmane isolate 4 revealed that this compound also contained three different ester groups, which could be tentatively identified as acetate, isovalerate, and an acetoxy isovalerate, based on the fragment ions displayed at m/z 321 (MH^+-160), 261 (MH^+-160-60), 219 (MH^+-160-102), and 159 (MH^+-160-102-60). However, the significant difference in intensity of the fragment ion at m/z 321, due to elimination of "acetoxy isovalerate," between this compound (20%) and Valmane

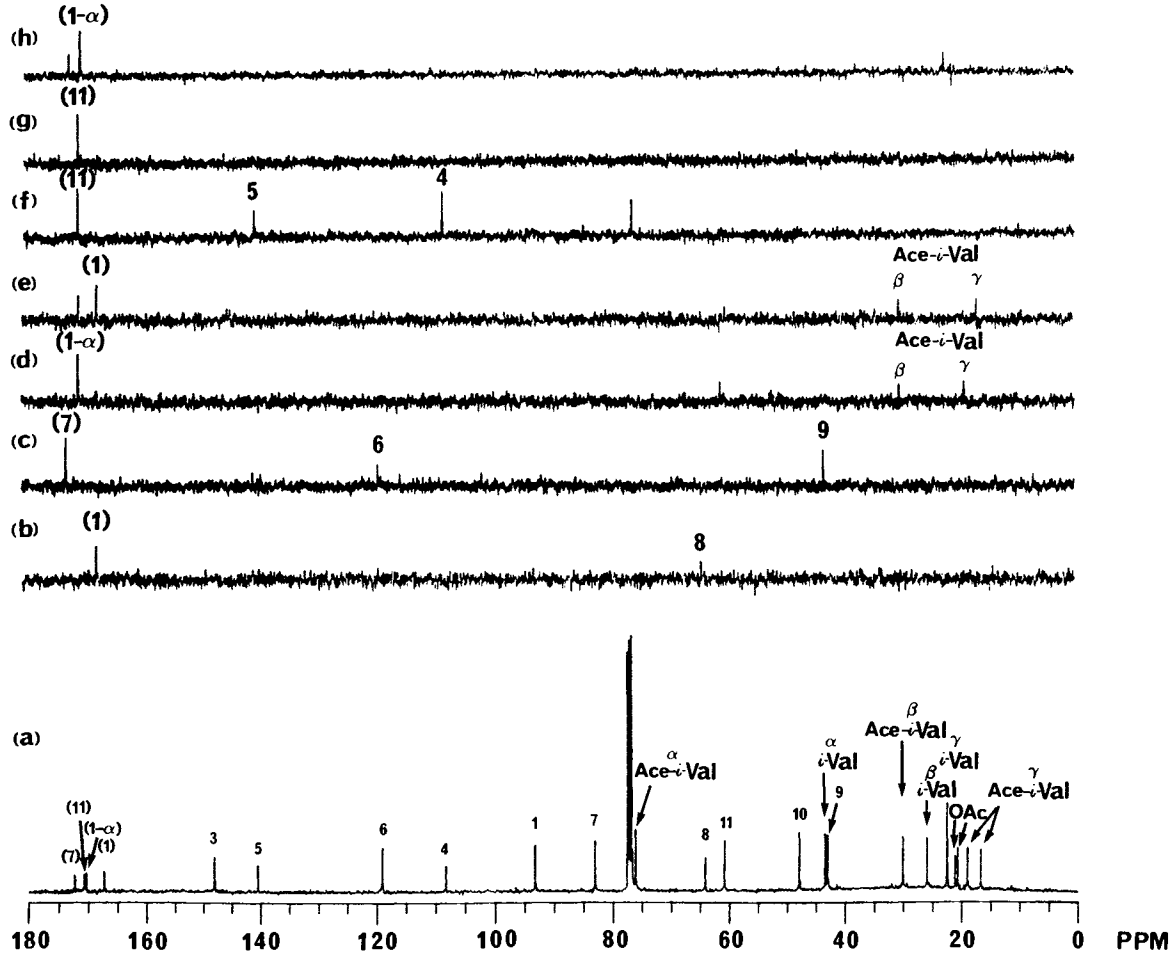

Fig. 4. Selective INEPT experiments of Valmane isolate 3: (a) broad-band decoupled spectrum; (b) irradiation of H-1, J = 3 Hz; (c) irradiation of H-7, J = 3 Hz; (d) irradiation of CH at 4.93 ppm, J = 3 Hz; (e) irradiation of CH at 4.93 ppm, J = 7 Hz; (f) irradiation of H-11, J = 3 Hz; (g) irradiation of acetate at 2.03 ppm, J = 7 Hz; (h) irradiation of acetate at 2.15 ppm, J = 7 Hz.

isolate 3 (7%) suggested that the location of the acetoxy isovaleryl group in these two compounds was different. The observation of an additional acetoxy signal at 1.95 ppm and two doublets at 2.90 and 2.84 ppm in the ^{1}H-NMR spectrum, as compared to those of valtrate, indicated that one of the acetoxy groups was at the β-position of the isovalerate. As a result of this substitution, it was also observed that two terminal methyl signals shifted downfield to 1.49 and 1.48 ppm due to the introduction of the electronegative substituent. The presence of a quaternary carbon at 79.22 ppm, a methylene carbon at 44.02 ppm, and two methyl carbons at 26.74 and 26.66 ppm in the ^{13}C-NMR spectrum provided additional evidence for this structure assignment.

Selective INEPT experiments (Fig. 5) showed that separate irradiation of H-11 (Fig. 5d) and the downfield acetoxy signal at 2.04 ppm (Fig. 5e) resulted in the enhancement of the same carbonyl carbon at 170.85 ppm, indicating that the downfield acetoxy group was at C-11. Separate irradiation of H-7 (Fig. 5c) and the center of two doublets (at 2.90 and 2.84 ppm) at 2.87 ppm (Fig. 5g) enhanced the same carbonyl carbon at 169.49 ppm and irradiation of the upfield acetoxy signal at 1.95 ppm enhanced the carbonyl carbon at 170.35 ppm (Fig. 5f); the acetoxy isovaleryl group was therefore at C-7 and the assignment of these two carbonyl carbons could be made unambiguously. It was found that previous assignment of these two carbons made by Thies *et al.* (11) should be reversed. By irradiating H-1, it was observed that the carbonyl carbon at 170.30 ppm was enhanced (Fig. 5b), revealing that the remaining ester group (isovalerate) was at C-1. The structure of Valmane isolate **4** thus could be established as 7-β-acevaltrate. This represents the first definitive clarification that the "acevaltrate" component of Valmane is not a single compound. It is noteworthy that Thies *et al.* (11) assigned 7-β-acevaltrate as the principal "acevaltrate" of *V. wallichii*. However, we obtained a larger quantity of 1-α-acevaltrate (vs 7-β-) from *V. wallichii*-derived Valmane (ratio of 1-α-:7-β-acevaltrate is about 10:1).

Comparison of the ^{1}H-NMR data of Valmane isolate **5** with those of valtrate revealed that this isolate was devoid of an acetyl moiety. However, the presence of a β-hydroxy isovaleryl substituent, which showed two characteristic signals at 2.47 and 1.25 ppm for the corresponding methylene and methyl protons, was evident. The other two ester groups were deduced to be isovalerate and β-methyl valerate based

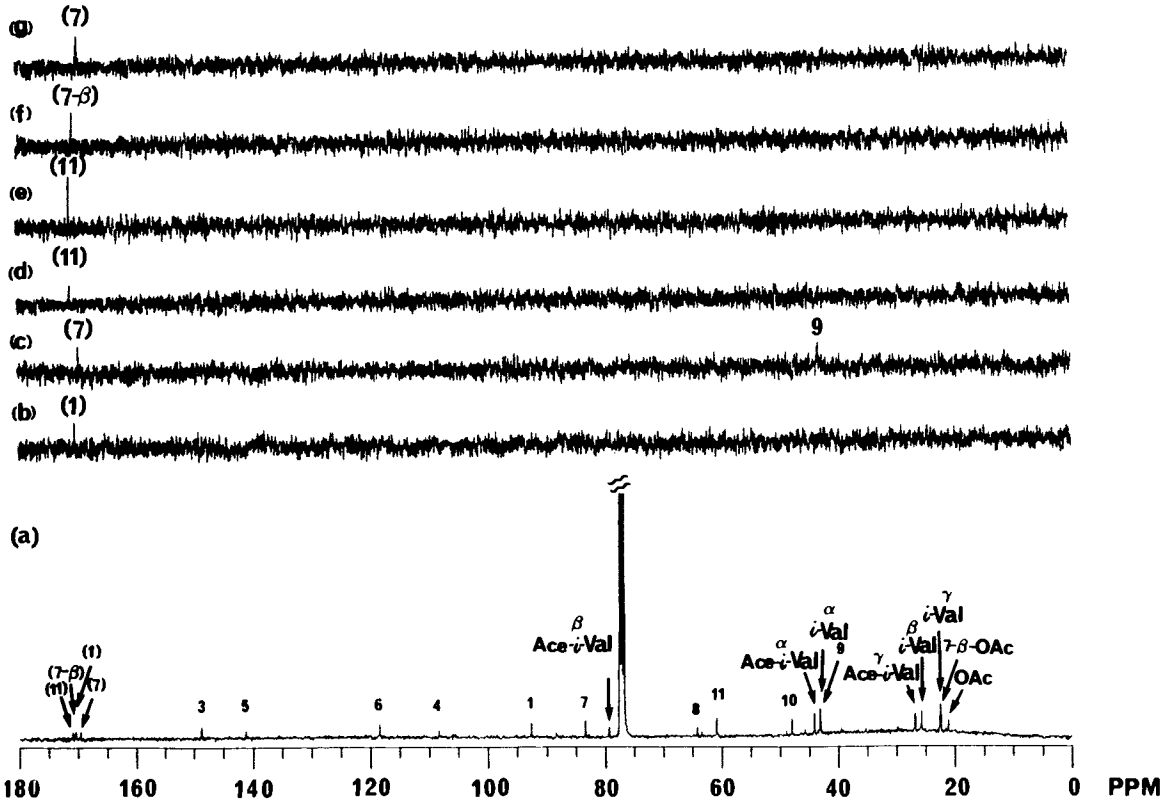

Fig. 5. Selective INEPT experiments of Valmane isolate 4: (a) broad-band decoupled spectrum; (b) irradiation of H-1, J = 3 Hz; (c) irradiation of H-7, J = 3 Hz; (d) irradiation of H-11, J = 3 Hz; (e) irradiation of acetate at 2.04 ppm, J = 7 Hz; (f) irradiation of acetate at 1.95 ppm, J = 7 Hz; (g) irradiation of CH_2 at 2.87 ppm, J = 5.5 Hz.

on interpretive integration of the MS, ^{1}H- and ^{13}C-NMR data. Detailed examination of the spectral data, however, revealed that this isolate was an unresolvable mixture of two compounds, **5a** and **5b**, in the ratio 2:1, with a one-carbon difference in one of the substituents, namely, one of the compounds contained one β-hydroxy isovaleryl and two isovaleryl groups in the molecule, whereas the other contained an isovaleryl, β-methyl valeryl, and β-hydroxy isovaleryl group in the molecule. Although the expected pseudo-molecular ions at *m/z* 481 and 495 for these two compounds, **5a** and **5b**, were not observed, the presence of prominent fragment ions at *m/z* 393 (495–isovalerate), 379 (495–β-methyl valerate and 481–isovalerate), 377 (495–β-hydroxy isovalerate), 363 (481–β-hydroxy isovalerate), 277 (495–isovalerate–β-methyl valerate and 481–two isovalerates), 275 (495–isovalerate–β-hydroxy isovalerate), 261 (481–isovalerate–β-hydroxy isovalerate and 495–β-hydroxy isovalerate–β-methyl valerate), and 159 (495–isovalerate–β-methyl valerate–β-hydroxy isovalerate and 481—two isovalerates–β-hydroxy isovalerate) confirmed this observation. According to Popov's empirical rules for $\Delta^{3,5}$-diene valepotriates (12), the substituents at C-1 and C-11 are eliminated mainly as radicals. The fragment ions at *m/z* 380 and 364 for compound **5a** and 394 and 378 for compound **5b** were thus expected to be more intense than those at *m/z* 379 and 363 for **5a** and 393 and 377 for **5b**, respectively. However, the opposite trend observed for these two compounds indicated that it is impossible to determine unambiguously the locations of these ester groups at C-1, C-7, or C-11 merely from the comparison of the relative intensity of those fragment ions resulting from elimination of acyloxy radical vs the corresponding acid.

In order to obtain the molecular ions for **5a/5b**, FAB-MS, in the positive and negative ion modes was also performed. Due to the lability of these two compounds, the expected pseudo-molecular ions were not observed. However, three major fragment ions at *m/z* 103 (isovalerate + H^+), 117 (β-methyl valerate + H^+), and 119 (β-hydroxy isovalerate + H^+) in the positive FAB-mass spectrum provided the evidence that **5a/5b** possessed three different substituents. The corresponding fragment ions at *m/z* 101 (isovalerate–H^+), 115 (β-methyl valerate–H^+), and 117 (β-hydroxy isovalerate–H^+) in the negative FAB-mass spectrum further confirmed this observation. Furthermore, the presence of two prominent fragment ions at *m/z* 261 and 159 in the positive FAB-mass spectrum, due to elimination of two and three ester groups, respectively, revealed that **5a/5b** had the same skeleton as valtrate (**1**).

To determine the locations of these ester groups, selective INEPT experiments were performed on this isolate (Fig. 6), even though it was an inseparable mixture. Irradiation of the methylene singlet at 2.47 ppm resulted in the enhancement of the carbonyl carbon at 172.68 ppm (Fig. 6e) [the same carbon which was enhanced when H-11 was irradiated (Fig. 6d)], demonstrating that the β-hydroxy isovalerate was at C-11 in both compounds. For compound **5a**, which has

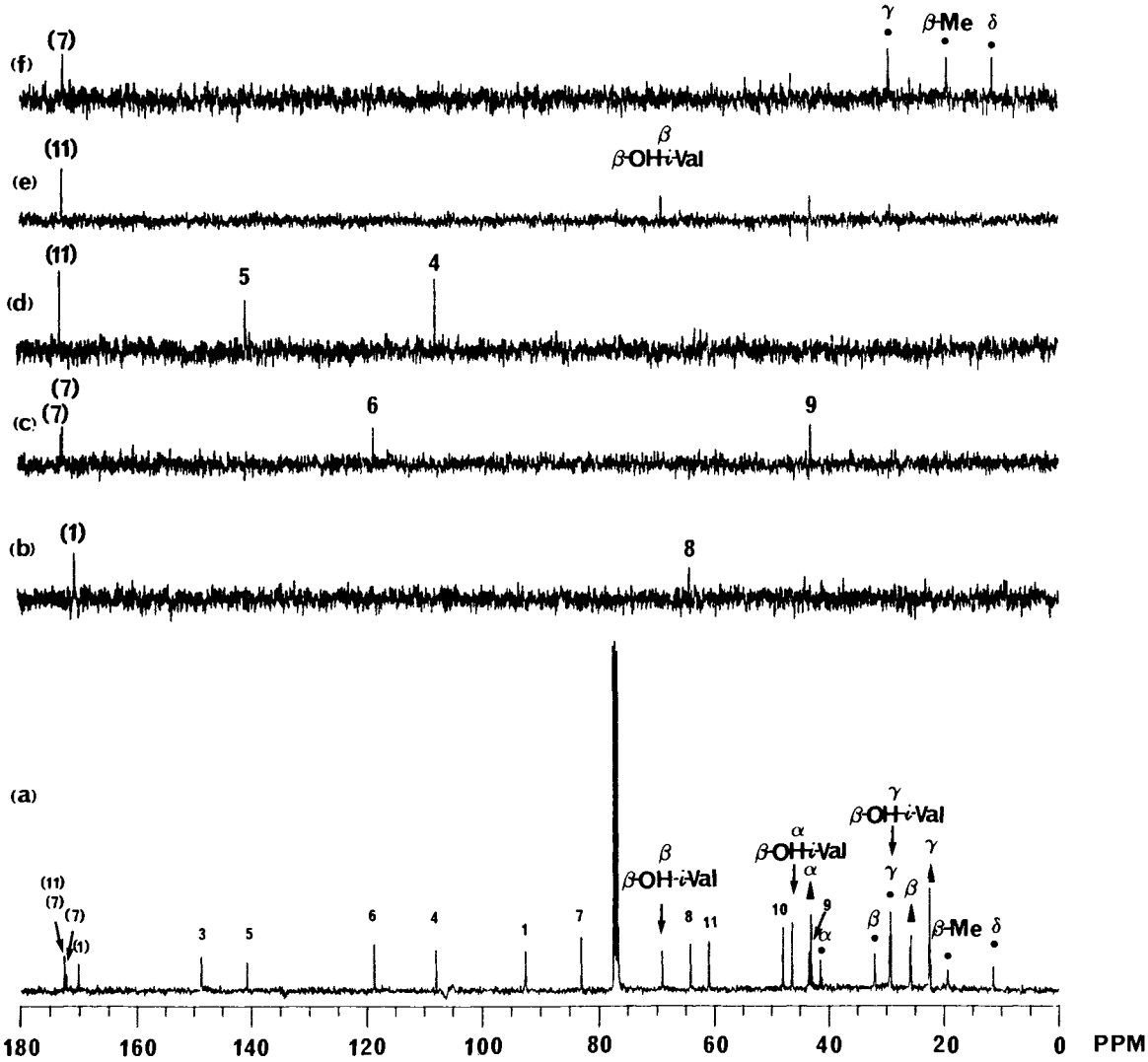

Fig. 6. Selective INEPT experiments of Valmane isolate 5: (a) broad-band decoupled spectrum; (b) irradiation of H-1, J = 3 Hz; (c) irradiation of H-7, J = 3 Hz; (d) irradiation of H-11, J = 3 Hz; (e) irradiation of CH_2 at 2.47 ppm, J = 7 Hz; (f) irradiation of CH at 1.84 ppm, J = 7 Hz. ● and ▲ are the carbons belonging to β-methyl valerate and isovalerate, respectively.

one β-hydroxy isovaleryl and two isovaleryl groups, these two isovaleryl groups can therefore be assigned to C-1 and C-7.

For compound **5b**, with three different esterifying substituents (isovalerate, β-methyl valerate, and β-hydroxy isovalerate), additional selective INEPT experiments involving H-1 and H-7 were required. Since irradiation of H-7 resulted in the enhancement of two carbonyl carbons at 172.68 and 172.43 ppm (Fig. 6c), and irradiation of H-1 enhanced only the carbonyl carbon at 170.31 ppm (Fig. 6b), an isovalerate group must be located at C-1 in both compounds, and a β-methyl valerate at C-7 in the latter compound **5b**. Due to the fortuit chemical shift equivalence of the two carbonyl carbons which are attached to C-7 in **5b** and C-11, it was observed that separate irradiation of H-7 (Fig. 6c) and H-11 (Fig. 6d) resulted in the enhancement of the same carbon signal. It was thus rationalized that in compound **5a**, which bears an isovalerate at C-7, this carbonyl carbon must exhibit a different chemical shift from that in **5b** which bears a β-methyl valerate at the same position. This explains why when H-7 was irradiated, two carbonyl carbon signals were enhanced. To assign these two carbonyl carbons unambiguously, further irradiation was performed on the methine proton of β-methyl valerate (which appeared at 1.84 ppm), showing that the carbonyl carbon at 172.68 ppm was enhanced significantly (Fig. 6f). This result demonstrated that the carbonyl resonance of the compound bearing the β-methyl valeryl group at C-7 was more downfield than that of the compound bearing the isovaleryl group at C-7. The structures of these isolates were thus determined to be as shown in Fig. 1. In Table I a comparison is given of the ^{13}C chemical shifts of the carbonyl carbons in the valtrate derivatives isolated from Valmane tablets. As can be seen, while some trends in relative shifts are evident with well-established esterifying groups, the introduction of unusual ester groups produces some unpredictable effects on the

Table I. Comparison of the ^{13}C Chemical Shifts of the Carbonyl Carbons in the Valtrate Derivatives **1**, **3**, **4**, **5a**, and **5b**

Compound	C-1	C-7	C-11	Other CO
1	170.19 IV[a]	172.31 IV	170.72 A	—
03	167.44 AAIV	172.42 IV	170.83 A	170.51
4	170.30 IV	169.49 BAIV	170.85 A	170.35
5a	170.31 IV	172.43 IV	172.68 BHIV	—
5b	170.31 IV	172.68 BMV	172.68 BHIV	—

[a] IV, isovaleryl; A, acetyl; AAIV, α-acetoxyisovaleryl; BAIV, β-acetoxyisovaleryl; BHIV, β-hydroxyisovaleryl; BMV, β-methylvaleryl.

chemical shift of the carbonyl carbons, thereby requiring a direct method for their unambiguous placement, such as the selective INEPT NMR technique described here.

CONCLUSIONS

Valmane tablets contain not three, but at least six, valepotriate derivatives. In this paper we have provided details of the unambiguous structure assignment of each of these constituents using the selective INEPT nmr technique. The structures of valtrate (**1**) and didrovaltrate (**2**) were conclusively demonstrated, and the "acevaltrate" component was shown to be a mixture of 1-α-acevaltrate (**3**) and 7-β-acevaltrate (**4**). Two new valtrate derivatives were isolated as a characterizable mixture of **5a** and **5b**.

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REFERENCES

1. M. Grieve. *A Modern Herbal, Vol. II,* Hafner, New York, 1959 (reprinted; originally published in 1931), pp. 824–830.
2. C. Hobbs. Valerian (*Valeriana officinalis*)—a literature review. *HerbalGram* No. **21**:19–34 (1989).
3. P. J. Houghton. The biological activity of valerian and related plants. *J. Ethnopharmacol.* **22**:121–142 (1988).
4. G. E. Trease and W. C. Evans. *Pharmacognosy,* 11th ed., Baillière Tindall, London, 1978, pp. 460–462.
5. V. E. Tyler. Plight of plant-drug research in the United States today. *Econ. Bot.* **33**:377–383 (1979).
6. V. E. Tyler. *The Honest Herbal: A Sensible Guide to the Use of Herbs and Related Remedies,* George F. Stickley, Philadelphia, 1982, pp. 17–18, 224–225.
7. V. E. Tyler, L. R. Brady, and J. E. Robbers. *Pharmacognosy,* 9th ed., Lea & Febiger, Philadelphia, 1988, p. 491.
8. *Rote Liste 1990,* Hypnotika/sedativa. Bundesverband der Pharmazeutischen Industrie e.V., Editio Cantor (Aulendorff/Württ), Berlin/Munich, group 48.
9. J. E. F. Reynolds (ed.). *Martindale: The Extra Pharmacopoeia,* 29th ed., Pharmaceutical Press, London, 1989, p. 1628.
10. O. Sticher. Plant mono, di- and sesquiterpenoids with pharmacological and therapeutical activity. In H. Wagner and P. Wolff (eds.), *New Natural Products and Plant Drugs with Pharmacological, Biological or Therapeutical Activity,* Springer, New York, 1977, pp. 137–176.
11. P. W. Thies, E. Finner, and S. David. On the active agents of valerian. XIV. Assignment of type and location of the acyloxy substituents in valepotriates via C13-NMR spectroscopy. *Planta Med.* **41**:15–20 (1981).
12. S. S. Popov and N. V. Handjieva. Mass spectrometry of valepotriates. *Biomed. Mass Spectrom.* **6**:124–128 (1979).
13. P. W. Thies. Die Konstitution der Valepotriate. Mitteilung über die Wirkstoffe des Baldrians. *Tetrahedron* **24**:313–347 (1968).
14. A. Bax. Structure determination and spectral assignment by pulsed polarization transfer via long-range ^{1}H–^{13}C coupling. *J. Magn. Reson.* **57**:314–318 (1984).
15. G. A. Cordell. NMR techniques for the structure elucidation and conformational analysis of natural products. *Kor. J. Pharmacog.* **19**:153–169 (1988).
16. G. A. Cordell. Selective INEPT spectroscopy—a powerful tool for the spectral assignment and structure elucidation of natural products. *Phytochem. Anal.* **2**:49–59 (1991).
17. H. Wagner, S. Bladt, and E. M. Zgainski. *Plant Drug Analysis: A Thin-Layer Chromatography Atlas,* Springer, New York, 1984, pp. 263–267.
18. P. W. Thies. Über die Wirkstoffe des Baldrians 2: Zur Konstitution der Isovaleriansäurester Valepotriat, Acetoxyvalepotriat und Dihydrovalepotriat. *Tetrahedron Lett.* **11**:1163–1170 (1966).
19. W. Foerster, H. Becker, and E. Rodriguez. HPLC analysis of valepotriates in the North American genera *Plectritis* and *Valeriana. Planta Med.* **50**:7–9 (1984).
20. H. Wagner and K. Jurcic. On the spasmolytic activity of *Valeriana* extracts. *Planta Med.* **37**:84–86 (1979).
21. C. Bounthanh, C. Bergmann, J. P. Beck, M. Haag-Berrurier, and R. Anton. Valepotriates, a new class of cytotoxic and antitumor agents. *Planta Med.* **41**:21–28 (1981).
22. W. von der Hude, M. Scheutwinkel-Reich, R. Braun, and W. Dittmar. In vitro mutagenicity of valepotriates. *Arch. Toxicol.* **56**:267–271 (1985).
23. M. Tortarolo, R. Braun, G. E. Hübner, and H. R. Maurer. *In vitro* effects of epoxide-bearing valepotriates on mouse early hematopoietic progenitor cells and human T-lymphocytes. *Arch. Toxicol.* **51**:37–42 (1982).
24. C. Bounthanh, L. Richert, J. P. Beck, M. Haag-Berrurier, and R. Anton. The action of valepotriates on the synthesis of DNA and proteins of cultured hepatoma cells. *Planta Med.* **49**:138–142 (1983).
25. G. Tittel, V. M. Chari, and H. Wagner. HPLC-Analysis of *Valeriana mexicana* extracts. *Planta Med.* **34**:305–310 (1978).