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# STRUCTURES OF MELLEOLIDES B–D, THREE ANTIBACTERIAL SESQUITERPENOIDS FROM *ARMILLARIA MELLEA**

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**Key Word Index**—*Armillaria mellea*; Basidiomycete; secondary metabolites; protoilludane derivatives; structural determination.

**Abstract**—The structures of melleolides B–D, three new protoilludene sesquiterpenoid O-methylorsellinates isolated from a culture of *Armillaria mellea*, have been elucidated on the basis of chemical and spectral data.

## INTRODUCTION

As a part of our studies on fungal metabolites, we have isolated melleolides B–D (**1–3**) from cultures of a strain of *Armillaria mellea*, a Basidiomycete which is known to produce chemical defence metabolites possessing antibacterial activity [1]. These compounds, which have an unusual hydroxylation pattern at C-10 and C-13, are new members of the protoilludane sequiterpenoids, a group of fungal metabolites first isolated from *Clitocybe illudens* [2]. It is suggested that they are derived from a 'protoilludane' intermediate arising from farnesol *via* humulene.

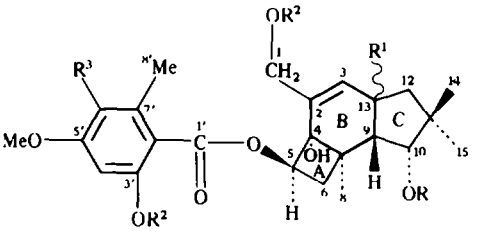
The first compound of this type to be isolated from *A. mellea* was armillol orsellinate [3, 4] followed by melleolide [5], armillarin and armillaridin [6], and judeol and 4-*O*-methylmelleolide [7]. In all cases the structures were established by X-ray analysis. In this paper, we report the isolation of melleolides B–D, aromatic esters of different derivatives of protoilludene, and their structural assignment on the basis of chemical transformations and $^1$H and $^{13}$C NMR spectroscopy.

## RESULTS AND DISCUSSION

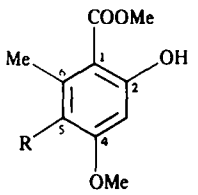
When *A. mellea* was grown on glucose–yeast extract–agar medium (GYA) in Roux flasks for 4 weeks, two main metabolites (**1** and **2**) were produced. Compound **1** was obtained as a glassy solid, mp 42–45°; $[\alpha]_D = +19.6°$ (*c* 0.6; MeOH). Its IR and UV spectra established the presence of an ester function (1645 cm$^{-1}$) and a substituted aromatic ring respectively. Its molecular formula ($C_{24}H_{32}O_7$) was determined by CI/MS ($[M]^+$, *m/z* 432). A major fragmentation ion at *m/z* 183 corresponded to the *O*-methylorsellinate moiety. The $^1$H and $^{13}$C NMR data, which are summarized in Tables 1 and 2, confirmed the proposed formula.

The presence of the *O*-methylorsellinate moiety was deduced from the following results: the 300.13 MHz $^1$H NMR spectrum of compound **1** exhibited one chelated phenolic hydroxy proton at $\delta_H$ 11.60 (OH-3'), two *meta* coupled aromatic protons at $\delta_H$ 6.32 and 6.25, one methoxy group at $\delta_H$ 3.79 and one aromatic methyl group at $\delta_H$ 2.37, while the 75.47 MHz $^{13}$C NMR spectrum showed the presence of one ester carbonyl carbon atom at $\delta_C$ 171.0 (C-1') and a tetrasubstituted benzene ring, besides two methyl carbon atoms, one of which was oxygen-bearing, at $\delta_C$ 55.3 and 24.0 respectively. In particular, the proton-bearing carbon atom at $\delta_C$ 99.1 was assigned to C-4' because it presented a three-bond coupling constant to the chelated 3'-hydroxy proton [$^3J$ (CH) = 6.5 Hz] [8].

NOE experiments (Table 3) located the methoxyl



**1** R = R² = R³ = H, R¹ = Hβ
**2** R = R² = R³ = H, R¹ = OH
**3** R = R² = H, R¹ = OH, R³ = Cl
**4** R = R² = Ac, R¹ = R³ = H
**5** R = R³ = H, R¹ = OH, R² = Ac
**6** R = R² = Ac, R¹ = OAc, R³ = H



**7** R = H
**8** R = Cl

*Part XIV in the series "Secondary Mould Metabolites". For Part XIII see Arnone, A., Camarda, L., Merlini, L. and Nasini, G. (1985) *J. Chem. Soc. Perkin Trans.* 1, 1387.

Table 1. $^{13}C$ NMR data of compounds 1, 2 and 3 (75.47 MHz, $CDCl_3$, TMS as int. standard)

| C | 1 | 2 | 3 |
|---|---|---|---|
| 1 | 65.6 *t* (143)* | 64.8 *t* (144) | 64.9 *t* |
| 2 | 133.4 *s* | 135.1 *s* | 135.0 *s* |
| 3 | 135.4 *d* (153) | 134.0 *d* (155) | 134.0 *d* |
| 4 | 76.5 *s* | 77.3† *s* | ‡ |
| 5 | 76.7 *d* (158) | 74.4 *d* (158) | 75.2 *d* |
| 6 | 33.0 *t* (138) | 32.3 *t* (140) | 32.5 *t* |
| 7 | 36.6 *s* | 36.7 *s* | 36.8 *s* |
| 8 | 21.2 *q* (126) | 21.3 *q* (126) | 21.3 *q* |
| 9 | 47.1 *d* (125) | 54.3 *d* (125) | 54.3 *d* |
| 10 | 82.5 *d* (149) | 82.9 *d* (149) | 82.6 *d* |
| 11 | 42.6 *s* | 40.8 *s* | 40.8 *s* |
| 12 | 44.9 *t* (129) | 55.9 *t* (129) | 55.9 *t* |
| 13 | 35.2 *d* (132) | 77.2† *s* | ‡ |
| 14 | 29.3 *q* (125) | 28.9 *q* (125) | 28.9 *q* |
| 15 | 23.9 *q* (125) | 23.5 *q* (125) | 23.5 *q* |
| 1′ | 171.0 *s* | 171.0 *s* | 170.4 *s* |
| 2′ | 105.3 *s* | 105.3 *s* | 106.8 *s* |
| 3′ | 165.7 *s* | 165.7 *s* | 162.7† *s* |
| 4′ | 99.1 *d* (161) | 99.1 *d* (161) | 98.8 *d* |
| 5′ | 164.2 *s* | 164.3 *s* | 159.9† *s* |
| 6′ | 111.3 *d* (161) | 111.4 *d* (162) | 115.9 *s* |
| 7′ | 143.0 *s* | 143.1 *s* | 139.5 *s* |
| 8′ | 24.0 *q* (128) | 24.1 *q* (129) | 19.5 *q* |
| OMe-5′ | 55.3 *q* (144) | 55.4 *q* (145) | 56.3 *q* |

*Values in parentheses are directly bonded ($^1J_{C,H}$) coupling constants in Hz.

†Assignments may be interchanged.

‡Obscured by $CDCl_3$.

group at C-5′, because its irradiation resulted in enhancement of both H-4′ and H-6′, and the aromatic methyl group at C-7′, because its irradiation produced enhancement of H-6′. In addition the three-bond coupling constant between H-5 and the carboxylic carbon atom C-1′ [$J$ (CH) = 2 Hz] proved the attachment point of this ester moiety was at C-5.

Methanolysis of compound 1 gave the expected methyl-2-hydroxy-4-methoxy-6-methylbenzoate (7), whose physical and spectroscopic properties were fully compatible with those reported [9]. The remainder of the signals of the $^1H$ and $^{13}C$ NMR spectra, which along with those of the *O*-methylorsellinate moiety were correlated by specific $^1H$ low-power heteronuclear decouplings were all in agreement with a protoilluden-tetraol skeleton. In fact the $^{13}C$ NMR spectrum revealed the presence of two olefinic carbon atoms belonging to a trisubstituted double bond (C-2 and C-3), and four oxygen-bearing (C-1, C-4, C-5 and C-10), three methyl (C-8, C-14 and C-15), two methylene (C-6 and C-12), two methine (C-9 and C-13) and two quaternary (C-7 and C-11) $sp^3$-hybridized carbon atoms. In addition, the magnitude of the directly bonded carbon 13-proton coupling constants ($^1J_{C,H}$) of 158 and 138 Hz exhibited by C-5 and C-6 strongly suggested that these two carbon atoms were part of the cyclobutane ring A [10].

The $^1H$ NMR spectrum showed an ABX system due to H-5 and $H_2$-6 which represented the cyclobutane ring protons. All of these possessed no vicinal coupling constants, thereby indicating that they must be adjacent to quaternary carbon atoms. $H_3$-8 which was W-type long-range coupled to H-6$\beta$ and gave an NOE to H-9 and H-10 (Table 3), was placed at C-7. In addition its irradiation produced NOE to H-5, thus establishing the relative configuration at C-5 and C-7. The two geminally coupled protons at $\delta_H$ 4.25 and 3.92 which shifted downfield after the formation of the triacetate 4 upon treatment of 1 with acetic anhydride–pyridine and showed allylic and homoallylic coupling constants to H-3 and H-13 respectively, were assigned to $H_2$-1, the above data indicating that $CH_2OH$ was located on the trisubstituted double bond. Furthermore, H-13 presented vicinal coupling constants to H-3, H-9 and $H_2$-12 while the signal at $\delta_H$ 3.61 (H-10) which shifted downfield upon acetylation of OH-10, was coupled to H-9 and W-type long-range coupled to H-12$\beta$. The two singlet methyl protons at $\delta_H$ 0.99 and 1.05 were assigned to $H_3$-14 and $H_3$-15. Selective irradiation of $H_3$-15 resulted in a positive enhancement of H-10 and H-12$\alpha$ while $H_3$-14 showed W-long-range coupling to H-12$\alpha$ and NOE effect to H-9, H-10 and H-12$\beta$ providing conclusive evidence for the structure of rings B and C. The sterically hindered aliphatic hydroxy group at $\delta_H$ 4.01 which remained as the only unesterified hydroxy function, had to be placed at the quaternary C-4 carbon atom. Irradiation of H-13 caused an NOE on H-6$\beta$ and H-9 thus establishing the *cis* A/B and B/C ring junctions.

From the magnitude of coupling constants and NOE experiments 1 must possess a *cis-anti-cis* stereochemistry. Moreover we propose that ring C has an envelope shape with C-10 turned down, the H-10 being $\beta$ located in order to account for the small value of $J_{9,10}$ = 3.7 Hz.

Melleolide C (2), mp 78–80°, $[\alpha]_D$ +27.9° (*c* 0.5; $CHCl_3$), was the second, and most abundant *O*-methylorsellinate sesquiterpenoid isolated from the mycelium. In many cultures it was accompanied by metabolite 3, from which it was separated only by reversed phase (RP-C18) chromatography.

The $M_r$ of 2 ($C_{24}H_{32}O_8$), established by CIMS with ammonia (*m/z* 448), indicated the presence in 2 of one oxygen more than 1. The MS showed ions derived from loss of water at *m/z* 430 $[M-H_2O]^+$, 412 $[M-2H_2O]^+$ and 394 $[M-3H_2O]^+$ typical of neoilludol derivatives [11]. Furthermore there was a peak at $[M-Ar(OH)(OMe)MeCOO]^+$ belonging to the sequiterpenoid part of the molecule; other peaks at *m/z* 249 (base peak), 231 and 213 indicated the same sequential loss of water from the aliphatic part of melleolide C whilst peaks at *m/z* 183, 165 and 137 were due to the aromatic moiety. Melleolide C was identified as 13-hydroxymelleolide B by comparison of spectral parameters of 1 and 2.

The ($^1H$, $^1H$) connectivity pattern for proton resonances as well as the $^{13}C$ NMR data showed unambiguosly the presence of a hydroxy group at C-13. Upon acetylation, 2 gave the diacetate 5 and the tetracetate 6 (see Experimental), while methanolysis gave 7, whose attachment point was, like 1, proved by three-bond coupling constants of 2 Hz between H-5 and the carboxylic carbon atom. The NOEs observed on 2 (Table 3) and the NOE between H-5 and OH-4 in the diacetate 5 established the *cis* A/B ring junction but no rigorous experimental proof was found to elucidate the B/C junction.

The EIMS of melleolide D (3), the third metabolite, which was isolated in very poor yield, showed molecular ions at *m/z* 482/484 with a ratio indicating the presence of one chlorine atom and corresponding to the formula

Table 2. $^1$H NMR data of compounds 1–6 (300.13 MHz, $CDCl_3$, TMS as int. standard)

| H | 1 | 2 | 3 | 4 | 5 | 6 | J | 1 (Hz) | 2 (Hz)‖ |
|---|---|---|---|---|---|---|---|---|---|
| 1A | 4.25 | 4.29 (4.28)* | 4.31 | 4.76 | 4.85 | 4.84 | 1A, 1B | 12.3 | 13.6 |
| 1B | 3.92 | 3.93 (4.00) | 3.93 | 4.42 | 4.56 | 4.38 | 1A, 3 | 1.0 | 1.4 |
| 3 | 5.86 | 5.98 (6.08) | 5.98 | 5.89 | 5.94 | 6.73 | 1A, 13 | 2.0 | — |
| 5 | 5.70 | 5.65 (5.65) | 5.62 | 5.22 | 5.29 | 5.17 | 1B, 3 | 1.0 | 1.4 |
| 6α | 2.00 | 2.00 (1.86) | 2.00 | 2 | 2.12 | 2 | 1B, 13 | 1.0 | — |
| 6β | 1.67 | 2.18 (2.43) | 2.18 | 2 | 2.01 | 2 | 3, 9 | 0.8 | 1.2 |
| Me-8 | 1.38 | 1.35 (1.35) | 1.35 | 1.21‡ | 1.35 | 1.21‡ | 3, 13 | 2.5 | — |
| 9 | 2.28 | 2.44 (2.45) | 2.44 | 2.41 | 2.42 | 2.70 | 5, 6α | 8.3 | 8.4 |
| 10 | 3.61 | 3.71 (3.70) | 3.71 | 4.94 | 3.70 | 5.02 | 5, 6β | 9.1 | 9.1 |
| 12α | 1.49 | 1.98 (1.94) | 1.98 | 2 | 1.90 | 2 | 6α, 6β | 10.8 | 10.8 |
| 12β | 1.96 | 1.82 (1.85) | 1.82 | 2 | 1.84 | 2 | 6β, 8 | 0.8 | 0.4 |
| 13 | 2.85 | — — | — | 2.86 | — | — | 9, 10 | 3.7 | 4.2 |
| Me-14 | 0.99 | 1.16 (1.14) | 1.16 | 1.05‡ | 1.15 | 1.15‡ | 9, 13 | 9.4 | — |
| Me-15 | 1.05 | 0.98 (0.97) | 0.98 | 0.87‡ | 0.98 | 0.84‡ | 10, 12β | 0.8 | 1.2 |
| 4′ | 6.32 | 6.32 (6.32) | 6.41 | 6.46 | 6.46 | 6.46 | 12α, 12β | 13.4 | 13.8 |
| 6′ | 6.25 | 6.26 (6.32) | — | 6.62 | 6.61 | 6.62 | 12α, 13 | 5.0 | — |
| 8′ | 2.37 | 2.38 (2.44) | 2.51 | 2.35 | 2.37 | 2.32 | 12α, 14 | 0.8 | 0.8 |
| OMe-5′ | 3.79 | 3.79 (3.82) | 3.88 | 3.79 | 3.79 | 3.79 | 12β, 13 | 10.2 | — |
| | 11.60† | 11.57†(11.60)† | 11.17† | 3.54† | 3.41† | 3.75† | 4′, 6′ | 2.7 | 2.5¶ |
| | 4.01† | 3.98†(4.04)† | N.a. | 2.25§ | 1.80† | 2.25§ | 4′, 8′ | 0.7 | 0.5¶ |
| | 2.95† | 2.3† (3.95)† | N.a. | 2.06§ | 1.80† | 2.06§ | 6′, 8′ | 1.0 | 0.7¶ |
| | 2.08† | 2.3† (3.86)† | N.a. | 2.01§ | 2.26§ | 2.04§ | | | |
| | — | 2.3† (3.34)† | N.a. | — | 2.01§ | 1.98§ | | | |

*Values in parenthesis are chemical shifts in acetone-$d_6$.
†Chemical shift of OH resonances.
‡Assignments may be reversed.
§Chemical shift of $MeCO_2$ resonances.
‖Coupling constants obtained in acetone-$d_6$.
¶Coupling constants of 5.
N.a., Not assigned.

Table 3. Connectivities (%) established by NOE difference experiments*

| Proton irradiated | 1† | 2‡ | 5§ |
|---|---|---|---|
| H-3 | H-1B (4.3), H-12α (5.5), H-13 (3.6). | H-1B (5.6), H-12α (4.6). | H-1B (2.6), H-12α (3.1). |
| $H_3$-8 | H-5 (17.3), H-6α (5.8), H-9 (4.3), H-10 (9.8). | H-5 (20.6), H-6α (5.1), H-9 (4.8), H-10 (11.3). | H-5 (15.4), H-6α (3.5), H-9 (5.2), H-10 (10.9). |
| H-9 | H-6β (2.3), H-10 (9.1), H-13 (7.6). | | H-10 (5.6), $H_3$-14 (0.9). |
| H-13 | H-3 (5.1), H-6β (4.3), H-9 (6.8), H-12β (4.3). | | |
| $H_3$-14 | H-9 (11.9), H-10 (6.6), H-12β (1.6). | H-9 (10.1), H-10 (6.2), H-12β (2.7). | H-9 (10.6), H-10 (7.3), H-12α (0.9), H-12β (2.7). |
| $H_3$-15 | H-10 (3.8), H-12α (6.3). | H-10 (4.4), H-12α (4.6), H-12β (0.9). | H-10 (3.9), H-12α (3.2), H-12β (2.0). |
| $H_3$-8′ | H-6′ (10.7). | | H-6′ (8.8). |
| OMe-5′ | H-4′ (21.3), H-6′ (7.8). | | H-4′ (11.3), H-6′ (8.8). |

*NOE values reported have only qualitative significance.
†Run in $CDCl_3 + D_2O$.
‡Run in acetone-$d_6 + D_2O$.
§Run in $CDCl_3$.

$C_{24}H_{31}O_8Cl$. Comparison of the NMR and MS data of 2 and 3 indicated that these compounds shared the same basic structure, the only significant difference being the presence of a chlorine atom on the *O*-methylorsellinate ring. In fact the $^1$H NMR spectrum of 3 (Table 2) contained only one aromatic proton at $\delta_H$ 6.41 (H-4′) which was correlated with the proton-bearing carbon atom at $\delta_C$ 98.8, assigned to C-4′ because in the $^{13}$C NMR spectrum it presented a three-bond coupling constant to the chelated 3′-hydroxy proton ($^3J$ (CH) = 7.5 Hz) [8].

Methanolysis of melleolide D (3) afforded compound 8, which was identified on the basis of the following results.

Its mass spectrum showed molecular ions at $m/z$ 230/232 indicating the presence of the chlorine atom, and its $^1$H NMR spectrum was consistent with the proposed structure (see Experimental). Moreover irradiation of methoxy protons only produced enhancement of H-3 (17.2%) while no signal experienced NOE effect by irradiation of the aromatic methyl protons. These findings located the methoxy and the methyl groups at C-4 and C-6 respectively. As a consequence the chlorine atom must be placed at C-5. Melleolide D (3) is therefore the 6'-Cl derivative of melleolide C. The substances 1, 2 and 3 exhibited remarkable antibacterial activity against *Bacillus cereus* (ATCC 10702), *B. subtilis* (ATCC 6633) and *Escherichia coli* (ATCC 10536).

Work is in progress to identify the structures of the many other different melleolides isolated from the same fungus.

## EXPERIMENTAL

Mps are uncorr. Flash chromatographies were performed with Merck silica gel (0.040–0.063 mm) and TLC with Merck $HF_{254}$ silica gel. Unless otherwise indicated, the purity of products was checked by TLC, NMR and MS and deemed sufficient for the purposes of structural elucidation.

*Isolation and purification of metabolites.* A strain of *A. mellea* obtained from 'Centro C.N.R. di Studio sulla Micologia del Terreno, Torino', grown on glucose–yeast extract–agar medium (GYA—3%, 1%, 1.5%) in 50 Roux flasks was extracted twice with EtOAc after 4 weeks growth at 24°. The extract was dried ($Na_2SO_4$) and evapd to give a brown mixture of crude metabolites. The mixture was chromatographed on a column of silica gel using hexane–EtOAc (2:1) and purified further by prep. TLC using the same solvent. Two main fractions, 1 (50 mg) and 2 (100 mg) containing 3 as ~10% impurity, were isolated. The metabolites 2 and 3 were separated by reverse phase chromatography on RP-C18 using $Me_2CO$–$H_2O$ (2:1) as eluent.

*Melleolide B* (1). MS $m/z$: 432, 414, 233, 183, 165; UV $\lambda_{max}^{95\%\,EtOH}$ nm: 264 and 303 (ε 14 500, 6150); NMR: see Tables 1 and 2.

*Melleolide C* (2). MS (electron impact) $m/z$: 430 $[M-18]^+$, 412 $[M-36]^+$, 238, 181, 165 (base peak), 137; UV $\lambda_{max}^{95\%\,EtOH}$ nm: 264 and 303 (ε 14.700, 6300); IR $\nu_{max}^{nujol}$ $cm^{-1}$: 3430 (OH), 1645 (ester); NMR: see Tables 1 and 2.

*Melleolide D* (3). Mp 96–98° ($CH_2Cl_2$–hexane); MS $m/z$: 482/484, 464/466 $[M-18]^+$; NMR: see Tables 1 and 2.

*Melleolide B triacetate* (4). Compound 1 (20 mg) was dissolved in 0.5 ml of dry pyridine and treated with $Ac_2O$ (0.5 ml). The mixture was left to stand at room temp. for 24 hr, dissolved in $CHCl_3$ and treated with satd $NaHCO_3$ soln, $H_2O$, satd $KHSO_4$ soln, $H_2O$ and finally dried ($Na_2SO_4$). Evapn of the solvent gave 4, glassy solid, mp 57–60°; NMR: see Table 2.

*Methanolysis of 1, 2 and 3.* Each compound (20 mg) was treated with 5 ml of 0.5 N KOH in MeOH at 60°for 10 min; evapn of the solvent, dilution with $H_2O$, neutralization and extraction with $CH_2Cl_2$ gave after prep. TLC with hexane–EtOAc (9:1) from 1 and 2, compound 7, and from 3, compound 8.

*Methyl-2-hydroxy-4-methoxy-6-methylbenzoate* (7). Mp 64–66° (hexane); MS $m/z$: 196 $[M]^+$, 164 (base peak); $^1$H NMR (300 MHz, $CCl_4$): δ 2.47 (3H, *br s*, Me-6), 3.91 and 3.79 (6H, *s*, 2OMe), 6.19 (1H, *dq*, $J$ = 2.6 and 0.8 Hz, H-5), 6.23 (1H, *br d*, $J$ = 2.6 Hz; H-3), and 11.60 (1H, *s*, OH-2); (Me-6): H-5 (NOE = 11.7%); (OMe-4): H-3 (13.1%) and H-5 (5.1%).

*Methyl-2-hydroxy-4-methoxy-5-chloro-6-methylbenzoate* (8). Mp 133–136° (hexane); MS $m/z$ 230/232 $[M]^+$, 198/200 (base peak), 170/172, 155/157; $^1$H NMR (300 MHz, $CDCl_3$): δ 2.63 (3H, *s*, Me-6), 3.95 and 3.91 (6H, *s*, 2OMe), 6.41 (1H, *s*, H-3), 11.56 (1H, *s*, OH-2); (OMe-4): H-3 (NOE = 17.2%).

*Melleolide C acetates* 5 *and* 6. Compound 2 (20 mg) was acetylated as for 1 to give compound 5, mp 79–81° ($CH_2Cl_2$–hexane); $[\alpha]_D$ +2.87° (*c* 0.9; $CHCl_3$); the same sample was again acetylated with $Ac_2O$–pyridine at 60° for 3 hr. Evapn of the solvent and prep. TLC in hexane–EtOAc (2:1) gave the tetraacetyl derivative 6 as an oil; NMR: see Table 2.

*Biological tests.* Antibacterial activity was tested with paper disks soaked with 10, 50 or 500 μg of compounds 1, 2 and 3 and the test microorganisms were inoculated in sterilized agar in Petri dishes; the metabolites exhibited inhibition until 100 μg.

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