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Brief Report

Synthesis of fluorescently labeled pyrazole derivative induceing a triple response in *Arabidopsis* seedlings

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Supplementary material

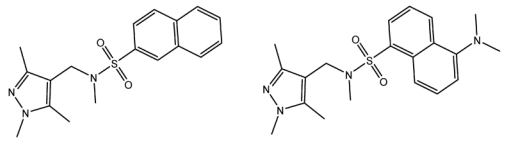
A fluorescent labeled pyrazole derivative with a dansyl moiety (**EH-DF**) was synthesized. Design of **EH-DF** was carried out by using a dansyl moiety to substitute the naphthalene moiety of the parent compound (**EH-1**). At a concentration of 30 μM, **EH-DF** displayed biological activity on inducing a triple response in *Arabidopsis* seedlings. Compared with the non-chemical treated control, the hypocotyl length of **EH-DF**-treated *Arabidopsis* seedlings was reduced from approximately 9.2±0.7 mm to 2.4±0.2 mm. The length of the roots was reduced from 1.7±0.1 mm to 1.0±0.1 mm, and the curvature of the hook of *Arabidopsis* seedlings increased from 60±16 degrees to 245±35 degrees. The maxim excitation wavelength and emission wavelength of **EH-DF** were 350 and 535 nm, respectively. Data obtained *via* fluorescent microscope analysis indicated that intensive fluorescent signals of **EH-DF** were observed in the shoot of *Arabidopsis* seedlings.

Keywords: plant hormone, ethylene, fluorescent probe.

Introduction

Plant hormones are important signal molecules involved in plant growth and development.[1)] Ethylene is a gaseous plant hormone that displays a variety of biological functions.[2–4)] Ethylene has been well characterized as a key hormone in the induction of seed dormancy release,[5)] the formation of the apical hook in dark-grown seedlings,[6)] flower opening,[7)] the control of fruit ripening,[8)] and senescence.[9)] Ethylene has also been implicated in the defense response to flooding[10)] and pathogen inflection.[11)] Despite ethylene's strong potential as a powerful agrichemical, its properties as a flammable gas made it difficult to use directly in many conditions. To meet the demands of developing new chemicals which are non-gaseous at normal atmosphere but with ethylene-like activity, we conducted a chemical screening of the compound library, and we found a pyrazole derivative (**EH-1**, the chemical structure is shown in Fig. 1) that displays promising activity for inducing a "triple response," an assay method widely used to determine ethylene activity, in *Arabidopsis* seedlings.[12)]

Fluorescence-labeled small molecules are powerful tools for biological study. They provide highly sensitive methods for monitoring complex cellular processes for research applications.[13)] To gain our understanding of the action mechanism of **EH-1**, we designed and synthesized a fluorescence-labeled **EH-1** derivative (**EH-DF**, the structure is shown in Fig. 1). In a previous work, we carried out a structure–activity relationship study of **EH-1**, and we found that the pyrazole moiety of **EH-1**



EH-1 **EH-DF**

Fig. 1. Chemical structures of **EH-1** and **EH-DF**

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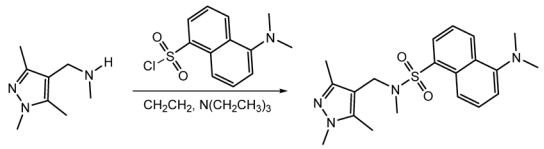

1 EH-DF

Scheme 1. General synthetic route for preparing **EH-DF**.

displayed an important effect on promoting the biological activity.[14] Based on this observation, the sulfonamide moiety of **EH-1** is a good candidate for introducing a fluorophore to **EH-1**, thereby developing fluorescence-labeled chemicals with ethylene-like biological activity. In the present work, we report the synthesis of *N*-methyl-*N*-[(1,3,5-trimethyl-1*H*-pyrazol-4-yl)methyl]-5-dimethylamino-1-naphthalenesulfonamide (**EH-DF**); we also determined the biological activity of **EH-DF** and discussed the application of **EH-DF**.

Materials and methods

1. General

^{1}H-NMR spectra were recorded with a JEOL ECP-400 spectrometer (Tokyo, Japan), with chemical shifts being expressed in ppm downfield from tetramethylsilane (TMS) as an internal standard. High-resolution electrospray ionization Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectra were recorded on an Exactive MS system (Thermo Fisher Scientific, Waltham, MA, USA).

2. Reagents

Chemicals for synthesis were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Reagents were of the highest grade commercially available. *N*-methyl-1-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)-methanamine (**1**) was purchased from Sigma-Aldrich.

3. Chemical synthesis

The general procedure for preparing *N*-methyl-*N*-[(1,3,5-trimethyl-1*H*-pyrazol-4-yl)methyl]-5-dimethylamino-1-naphthalenesulfonamide (**EH-DF**) was carried out by reacting *N*-methyl-1-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)methanamine with dansyl chloride in a condition as we described previously.[14] (yield: 33%), oil, ^{1}H NMR (400 MHz, $CDCl_3$); δ: 2.01 (s, 3H), 2.07 (s, 3H), 2.63 (s, 3H), 2.89 (s, 6H), 3.67 (s, 3Hs), 4.08 (s, 2H), 7.19 (d, J=7.3 Hz, 1H), 7.52–7.57 (m, 2H), 8.21 (d.d, J1=1.2, J2=7.3 Hz, 1H), 8.45 (d, J=8.6 Hz, 1H), 8.57 (d, J=8.6 Hz, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ 152.9, 148.2, 140.5, 135.1, 134.2 (x2), 131.4, 131.1, 130.8, 128.9, 124.2, 120.7, 116.7, 116.3, 111.5, 51.8, 45.6, 43.8, and 33.5. The HRMS-ESI calculated for $C_{20}H_{26}N_4O_2S$ [M+H]+ was 386.1776, and we found 386.1759.

4. Plant materials, growth conditions, and triple response assay

Wild-type *Arabidopsis* (ecotype Columbia) seeds were purchased from Lehle Seeds (Round Rock, TX, USA). A triple response assay was performed in a 24-well plate (Fukae Kasei Co., Ltd., Kobe, Japan). Stock solutions of all chemicals in this study were dissolved in dimethyl sulfoxide (DMSO) in designed growth media at 0.1% (v/v), as we described previously.[22]

*5. Determining the fluorescence spectra of **EH-DF***

The fluorescence spectra of **EH-DF** were measured using an F-4500 Fluorescence Spectrophotometer (Hitachi Co., Ltd., Tokyo, Japan). For each data point, 10 measurements were taken with the excitation slit and emission slit settings at 10 and 10 nm, respectively. Samples dissolved in the H_2O containing 0.1% DMSO were used.

6. Fluorescence microscopy

To image the cells in plant tissues, the plants were placed on slide glasses and mounted with cover slips. Bright and fluorescence images were captured with a BX51 fluorescence microscope (Olympus, Tokyo) equipped with a set of optical filters: WU-type (330–385 nm) and WIB-type (460–490 nm). Digital images were recorded with a DP70 (Olympus, Tokyo) digital camera.

Results

1. Chemistry

We have previously shown that the modifications of the sulfonamide moiety did not significantly influence the biological activity of **EH-1**.[14] Thus, introducing a dansyl moiety to **EH-1** represents a straightforward approach to developing fluorescence-labeled **EH-1**. Dansyl chloride (5-(dimethylamino)naphthalene-1-sulfonyl chloride) is a reagent that reacts with amines to produce stable blue or blue-green fluorescent sulfonamide adducts that display sufficient fluorescence properties. Moreover, the chemical structure of the target compound *N*-methyl-*N*-[(1,3,5-trimethyl-1*H*-pyrazol-4-yl)methyl]-5-dimethylamino-1-naphthalenesulfonamide (**EH-DF**) is similar to that of **EH-1**. Based on these observations, we thus designed the target compound by introducing a dansyl moiety to the **EH-1** (Fig. 1). The synthesis of **EH-DF** was carried out by reacting dansyl chloride with commercially available *N*-methyl-1-(1,3,5-trimethyl-1*H*-pyrazol-4-yl)methanamine (Sigma-Aldrich) in a condition we described previously (Scheme 1).[14]

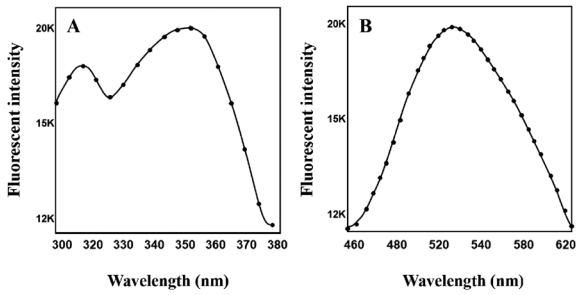

Fig. 2. Fluorescence spectra of **EH-DF.** The fluorescence spectra were recorded under conditions as described in the Experimental section. (A) Excitation spectra of **EH-DF** (10 μM) are shown. (B) Emission spectra of **EH-DF** (10 μM) are shown.

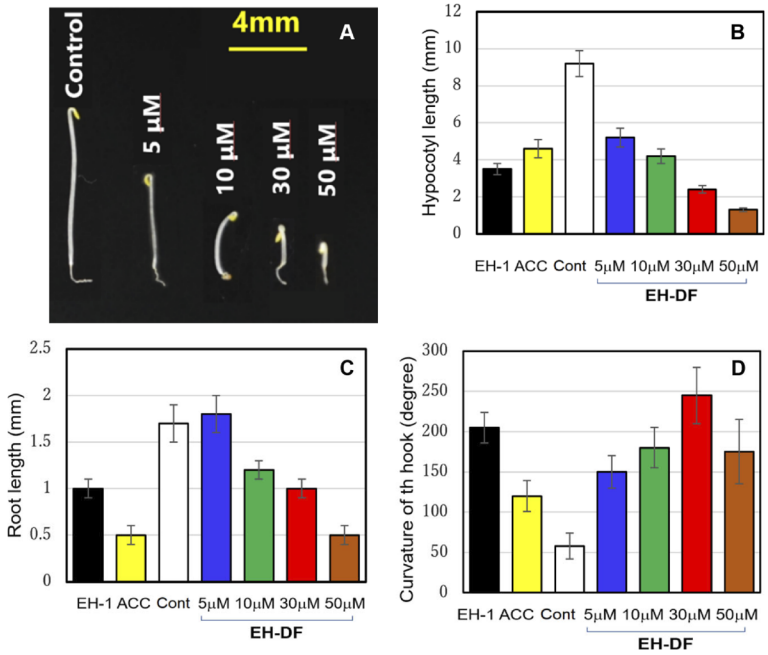

Fig. 3. Effect of **EH-DF** on inducing the morphology typical of a triple response in *Arabidopsis* seedlings. The triple responses of *Arabidopsis* seedlings were measured by determining the hypocotyl length, root length, and curvature of the hook. Approximately 50 seeds/well of *Arabidopsis* were grown for three days in the dark in a 24-well plate on medium containing 1/2 MS. The concentration of the positive control of **EH-1** (black bar) and ACC (yellow bar) were set at a final concentration of 10 μM, while using the DMSO mock treatment (white bar) as a control. The concentrations of **EH-DF** were set at 5 μM (blue bar), 10 μM (green bar), 30 μM (red bar), and 50 μM (brown bar). (A) The morphology typical of non-treated and chemically treated *Arabidopsis* seedlings are shown. From the left: control; second from the left: grown in the presence of 5 μM **EH-DF**; middle: grown in the presence of 10 μM **EH-DF**; second from the right: grown in the presence of 30 μM **EH-DF**; right: grown in the presence of 50 μM **EH-DF**. (B) Effect of **EH-DF** on the hypocotyl elongation of *Arabidopsis* seedlings. (C) Effect of **EH-DF** on the root elongation of *Arabidopsis* seedlings. (D) Effect of **EH-DF** on inducing exaggerated apical hook of *Arabidopsis* seedlings. Data obtained were determined from 11 seeds. Data are the means±S.E. obtained from 11 plants. All experiments were done three times to establish repeatability.

*2. Fluorescence properties of **EH-DF***

The fluorescence spectra of **EH-DF** are shown in Fig. 2. **EH-DF** displayed a maximal excitation wavelength at 350 nm and a maximal emission wavelength at 535 nm. At a concentration of 10 μM, **EH-DF** displayed sufficient fluorescence intensity of approximately 20000 in our experimental conditions.

*3. Triple response-inducing activities of **EH-DF** in* Arabidopsis *seedlings*

To determine the effect of **EH-DF** on inducing triple responses of *Arabidopsis* seedlings, we recorded the morphological characteristics of *Arabidopsis* seedlings that were grown in the dark for three days (Fig. 3A). First, we determined the effect of **EH-DF** on the hypocotyl elongation of *Arabidopsis* seedlings while using 1-aminocyclopropane-1-carboxylate (ACC, 10 μM) and **EH-I** (10 μM) as a positive control. As shown in Fig. 3B, the hypocotyl length of the non-chemical treated control was approximately 9.2±0.7 mm (the white bar), while the hypocotyl lengths of ACC- or **EH-1**-treated *Arabidopsis* seedlings were approximately 4.6±0.5 mm (the yellow bar) and 3.5 mm, respectively. This result indicated that ethylene and **EH-1** inhibit the hypocotyl elongation of *Arabidopsis* seedlings in our assay system. In terms of the biological activity of the synthesized **EH-DF**, we found that **EH-DF** reduced the hypocotyl length of *Arabidopsis* seedlings in a dose-dependent manner (Fig. 3B). The lengths of the hypocotyl of *Arabidopsis* seedlings were 5.2±0.5 mm (5 μM, blue bar), 4.2±0.4 mm (10 μM, green bar), 2.4±0.2 mm (30 μM, red bar), and 1.3±0.1 mm (50 μM, brown bar). Next, we determined the effect of **EH-DF** on the growth of *Arabidopsis* seedling roots. As shown in Fig. 3C, the root length of the non-chemically treated control was approximately 1.7±0.1 mm (the white bar), while the hypocotyl lengths of the positive controls of ACC (10 μM)- and **EH-1** (10 μM)-treated *Arabidopsis* seedlings were approximately 0.5±0.1 mm (the yellow bar) and 1.0±0.1 mm, respectively. We found that **EH-DF** reduced the root lengths of *Arabidopsis* seedlings in a dose-dependent manner (Fig. 3C). The lengths of the roots of *Arabidopsis* seedlings were 1.8±0.2 mm (5 μM, blue bar), 1.2±0. 1 mm (10 μM, green bar), 1.0±0.1 mm (30 μM, red bar), and 0.5±0.1 mm (50 μM, brown bar). Data obtained in the present work indicated that the activity of **EH-DF** on reducing the root elongation of *Arabidopsis* was weaker than that of **EH-1**. Finally, we determined the effect of **EH-DF** on the apical hook development. As shown in Fig. 3D, the curvature of the hook of the non-chemically treated control was approximately 60±16 degrees (the white bar), while the curvature of the hook of ACC (10 μM, the yellow bar)- and **EH-1**(10 μM)-treated *Arabidopsis* seedlings were approximately 119±19 degrees (the yellow bar) and 205±19 degree (the black bar), respectively. The curvatures of the hooks of **EH-DF**-treated *Arabidopsis* seedlings were found to be 150±20 degrees (5 μM, blue bar), 180±25 de-

grees (10 μM, green bar), 245±35 degrees (30 μM, red bar), and 175±40 degrees (50 μM, brown bar). This result indicated that a high concentration of **EH-DF** may have a negative effect on inducing the curvature of the hook of *Arabidopsis* seedlings. The asymmetric growth in the hypocotyl is the driving force for the apical hook curvature. The impairment of the apical hook as induced by treatment of 30 μM **EH-DF** is assumed to be due to its excessively inhibitory effect on hypocotyl growth, which results in the loss of the cell elongation for asymmetric growth.

Data obtained in the present work clearly indicate that **EH-DF** displays promising activity for reducing hypocotyl elongation as well as root growth of *Arabidopsis* seedlings in a dose-dependent manner. However, reducing the root elongation of *Arabidopsis* seedlings requires a high concentration of **EH-DF** (Fig. 3B). In addition, **EH-DF** displays promising activity for inducing the curvature of the *Arabidopsis* seedlings. Taking the above results together, we have chosen a 30 μM concentration of **EH-DF** for further fluorescence studies.

*4. Fluorescence imaging of **EH-DF**-treated* Arabidopsis *seedlings*

To determine the effect of **EH-DF** on the growth of *Arabidopsis* seedlings, we recorded the fluorescence images of **EH-DF**-treated *Arabidopsis* seedlings. As shown in Fig. 4, the blue-green fluorescence signal of **EH-DF** was significantly observed in the shoots of *Arabidopsis* seedlings (Fig. 4B). Merging the images captured under bright lights (Fig. 4A) and fluorescence microscopy (Fig. 4B) indicated that the fluorescence signals of **EH-DF** were intensively found in the upper/or lower parts of cotyledon cells (Fig. 4C). Interestingly, limited fluorescence signals of **EH-DF** were observed in root cells. To confirm whether these spatial distributions are special to **EH-DF**, we observed the fluorescence in *Arabidopsis* seedlings treated with a mock control or 5-(dimethylamino)-naphthalene-1-sulfonic acid (30 μM), the fluorophore of **EH-DF**. As shown in Fig. 4D and 4E, the mock control–treated *Arabidopsis* seedlings displayed dark blue fluorescence (Fig. 4D), while 5-(dimethylamino)naphthalene-1-sulfonic acid (30 μM)-treated *Arabidopsis* seedlings displayed green fluorescence (Fig. 4E) in cotyledon cells. Meanwhile, blue and green fluorescence signals were found in stem and root cells of the mock control- and 5-(dimethylamino)naphthalene-1-sulfonic acid (30 μM)-treated *Arabidopsis* seedlings, respectively (see supplemental information). In addition, as expected, 5-(dimethylamino)naphthalene-1-sulfonic acid (30 μM)-treated *Arabidopsis* seedlings did not show phenotypic characteristic of exaggerated apical hooks (Fig. 4D and 4E). These results indicate that **EH-DF** has a specific tissue- and cellular-distribution purpose in *Arabidopsis* seedlings, which could contribute to its physiological effects.

Because the chemical structures of **EH-1** and **EH-DF** are quite similar, it is possible that both compounds display the same mode of action for inducing a triple response in *Arabidopsis* seedlings. To verify this possibility, we tested the complementary effect of **EH-1** on **EH-DF**-induced triple response by the co-application of **EH-1** (15 μM) to **EH-DF** (15 μM), and the biological activities of the mixed compounds were determined. As shown in Fig. 4F, the co-application of **EH-1** to **EH-DF** enhanced the biological activity of **EH-DF**, which can be found by

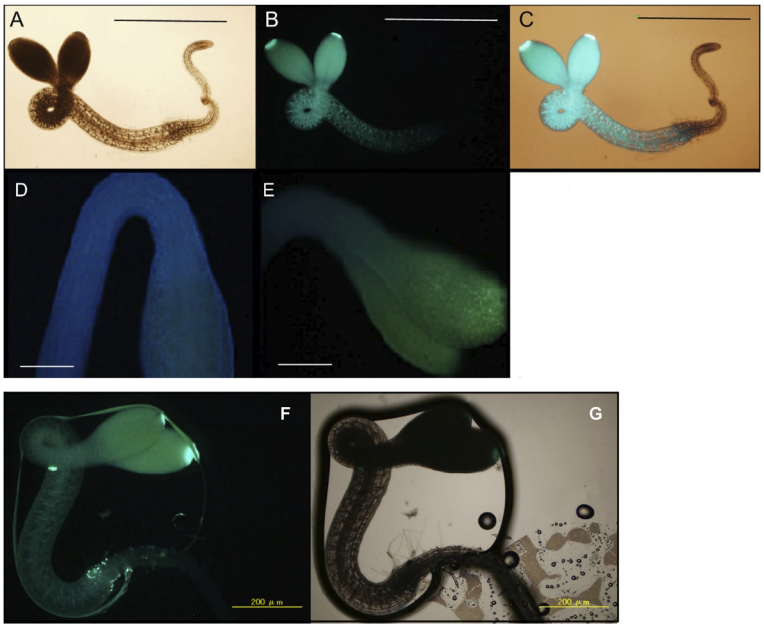

Fig. 4. Visualization of **EH-DF** in *Arabidopsis* seedlings. Approximately 50 seeds/well of *Arabidopsis* seedlings were grown for three days in the dark in a 24-well plate on medium containing 1/2 MS. (A) Image of **EH-DF** (30 μM)-treated *Arabidopsis* seedlings under bright light. (B) Fluorescence images of **EH-DF** (30 μM)-treated *Arabidopsis* seedlings. (C) A merger of A and B. (D) Fluorescence image of none-chemically treated *Arabidopsis* seedlings. (E) Fluorescence image of 5-(dimethylamino)naphthalene-1-sulfonic acid (30 μM)–treated *Arabidopsis* seedlings. (F) Fluorescence images of the co-application of **EH-DF** (15 μM)- and **EH-1** (15 μM)-treated *Arabidopsis* seedling. (G) A merger of fluorescence and bright light images. Fluorescence images were captured with a BX51 fluorescence microscope (Olympus, Tokyo) equipped with a set of optical filters—WU (330–385 nm) and WIB (460–490 nm). Scale bar: 1 mm.

comparing with the control (Fig. 4B, 30 μM **EH-DF**), while the fluorescence intensities observed in *Arabidopsis* seedlings were decreased (by comparing Fig. 4C and Fig. 4G). This result indicated that **EH-DF** may bind to the same target as **EH-1**.

Discussion

Our previous study identified **EH-1** as a non-gaseous chemical inducer of triple response,[12] which is expected to be applied in basic research and agriculture. However, the target and modes of action of **EH-1** are still elusive. To provide a strategy for studies on **EH-1**, we designed and synthesized a derivative (**EH-DF**) by introducing a fluorescent moiety that could facilitate tracing the chemical *in planta* (Fig. 1). The fluorescence of **EH-DF** can be excited and detected at 350 nm and 535 nm (Fig. 2). Morphogenic observations confirmed that **EH-DF** displays bioactivities similar to those of **EH-1** in triggering a triple response (Fig. 3), indicating their common mode of actions *in planta*.

We traced **EH-DF** by observing the fluorescence in *Arabidopsis* seedlings. Interestingly, we found a tissue-specific accumulation of fluorescence in the cotyledon and hypocotyl but not in the primary root (Fig. 4), while this fluorescence pattern and the exaggerated apical hook were not observed in seedlings treated by the mock control (autofluorescence) or fluorescent moiety of **EH-DF** (Fig. 4D, 4E, respectively). These results suggest a tissue-specific distribution of **EH-DF** that could contribute to its physiological effects in hypocotyl elongation and apical hook development. However, the reasons for **EH-DF**'s inhibition of the primary root growth despite the observance of no fluorescence locally in root remain elusive. For this issue, yet to be determined, we postulate possibilities that **EH-DF** could interact with a target protein specifically localized in the shoot, or **EH-DF** could be transported or metabolized in the root.

In addition to tissue specificity, we also noticed a cellularly polar distribution of **EH-DF** (Fig. 4), which is reminiscent of the distribution pattern of PIN FORMEDs (PINs), the transporters for polar auxin transports (PATs).[15] It has been well characterized that PINs mediate the PAT and are critical for apical hook development,[16] which was also observed in seedlings treated with **EH-1** and **EH-DF**. Thus, it will be interesting to investigate the relationship between **EH-DF** and PINs or other auxin-related components.

Taken together, although the target protein of **EH-1** still must be determined, we have developed **EH-DF** as a fluorescence tool to dissect the molecular modes of action. Data obtained from the present work clearly confirmed that **EH-DF** is a biofunctional mimic of **EH-1** and indicated a relationship between **EH-1** derivatives and auxin. Further investigation of the effects of **EH-1** and **EH-DF** in relationship with auxin may lead us to clarify their modes of action and target proteins.

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Electronic supplementary materials

The online version of this article contains supplementary material (Fig. S1), which is available at https://www.jstage.jst.go.jp/browse/jpestics/.

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