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Bioorganic & Medicinal Chemistry Letters 14 (2004) 3033–3036
Bioorganic & Medicinal Chemistry Letters

A new lead compound for abscisic acid biosynthesis inhibitors targeting 9-*cis*-epoxycarotenoid dioxygenase

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Received 17 March 2004; revised 14 April 2004; accepted 15 April 2004

Abstract—9-*cis*-Epoxycarotenoid dioxygenase (NCED), a key enzyme in abscisic acid (ABA) biosynthesis, cleaves the olefinic double bond of 9-*cis*-epoxycarotenoid. Several analogues of nordihydroguaiaretic acid (NDGA) were designed and synthesized, and their efficacy as inhibitors of NCED was examined. One of the synthesized compounds (**20**) was found to be an inhibitor of this enzyme, and inhibited ABA accumulation and stomatal closing, suggesting that **20** should be ABA biosynthesis inhibitor.

1. Introduction

Plants can respond to environmental stresses, such as drought, cold, and high salt, and can control aspects of their growth and development. One important regulator of these responses is plant hormone abscisic acid (ABA).[1–3] The endogenous concentration of ABA increases over 10-fold within a few hours of drought stress and decreases dramatically to normal levels following rehydration.[4] In higher plants ABA is synthesized via oxidative cleavage of epoxycarotenoids,[5–7] which is the key regulatory step in ABA biosynthesis in response to environmental stresses (Fig. 1).[8–10] The enzyme, 9-*cis*-epoxycarotenoid dioxygenase (NCED), catalyzes the cleavage of 9-*cis*-epoxycarotenoids to apocarotenoid (C_{25}) and xanthoxin (C_{15})[8] and is up-regulated by water-deficit stress.[11]

Fluridone and norflurazon have been used to identify ABA functions in plants as ABA biosynthesis inhibitors.[12–15] Both inhibit phytoene desaturase, which converts phytoene to phytofluene in the carotenoid biosynthesis pathway. Since carotenoids are the precursors of ABA in plants, carotenoid biosynthesis inhibitors should also prevent the biosynthesis of ABA.[13,16,17] However, the inhibition of carotenoid biosynthesis using fluridone and norflurazon causes lethal damage during plant growth because carotenoids play an important role in protecting photosynthetic organisms against photooxidation damage and absorb light energy in plants.[18] Therefore, the use of these phytoene desaturase inhibitors in the investigation of ABA functions is limited to narrow physiological aspects.

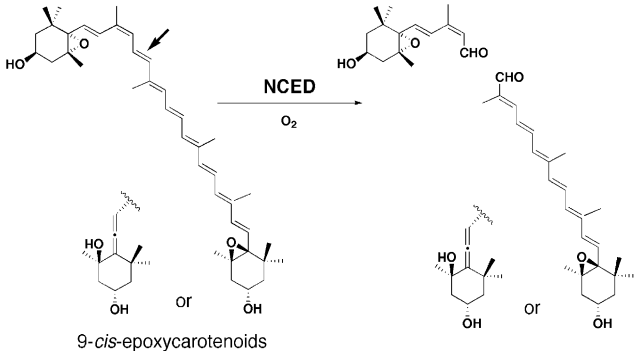

Figure 1. Cleavage of epoxycarotenoids by NCED.

Keywords: Abscisic acid; Plant hormone; Inhibitor; Carotenoid; Dioxygenase; Biosynthesis.
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doi:10.1016/j.bmcl.2004.04.035

In view of the indispensable nature of carotenoids and the importance of ABA functions in plants, it is worthwhile synthesizing and evaluating specific inhibitors of ABA biosynthesis that would be useful tools for functional studies of ABA biosynthesis and the effects of ABA in higher plants as was seen in the case of brassinosteroid biosynthesis inhibitors.[19–22] In this context, we started finding ABA biosynthesis inhibitors.

2. Results and discussion

In order to develop novel specific ABA biosynthesis inhibitors, NCED is an attractive target because it is the key regulatory enzyme in the ABA biosynthesis pathway.[23,24] Therefore, to find a lead compound for ABA biosynthesis inhibitor, firstly we tested the inhibitory activity of NDGA (nordihydroguaiaretic acid) against NCED in vivo because NDGA was reported to decrease ABA content in treated-plants.[25] An enzymaic test was performed according to the method described by Iuchi et al.[26] When NCED expressed in *E. coli* was incubated with 9′-*cis*-neoxanthin, the main products of this reaction were C_{25}-apocarotenoid and xanthoxin. To quantify the molar amounts of the products, the C_{25}-products were analyzed by HPLC using all-*trans*-violaxanthin as an internal standard. In this test we found that NDGA inhibited about 45% of NCED activity at 100 μM. Then we started the modification of the chemical structure of NDGA to increase its specificity as ABA biosynthesis inhibitor because NDGA has been reported to inhibit lipoxygenases and several events in cells[27,28] and therefore it is easily supposed that NDGA should not be a specific ABA biosynthesis inhibitor. Whitman et al. reported that the alkylation of the hydoxyl groups on the phenyl moiety of NDGA reduced its inhibitory activity against lipoxygenases.[29] On the basis of this observation, we anticipated that the alkylation of phenol group could reduce the lipoxygenase inhibitory activity of NDGA and therefore strengthen the specificity of NDGA derivatives to ABA biosynthesis inhibition. In our preliminary test, chemicals possessing nitrogen atom instead of carbon atom in the chain connecting two phenyl groups showed significant activity. Thus, in order to develop ABA biosynthesis inhibitors targeting NCED, a number of compounds were synthesized as summarized in Figures 2 and 3.[30–33] 4-{[2-(4-Hydroxyphenyl)ethylimino]methyl}benzene-1,2-diol (**1**) was prepared by the condensation of 4-(2-aminoethyl)phenol with 3,4-dihydroxybenzaldehyde (Fig. 2). Compounds **2** and **3** were prepared by the same method used for compound **1**. 4-{[2-(4-Hydroxyphenyl)ethylamino]methyl}benzene-1,2-diol (**4**) was produced from the reduction of compound **1** using sodium borohydride ($NaBH_4$). Compounds **5**, **6**, **11**, and **12** were prepared using the method described for compound **4**. The reaction of 4-[2-(3,4-dimethoxy-benzylamino)ethyl]-phenol (**5**) with iodomethane in the presence of NaH produced (3,4-dimethoxybenzyl)[2-(4-methoxy-phenyl)-ethyl]methylamine (**7**). Compound **8** was prepared by the method described for compound **7**.

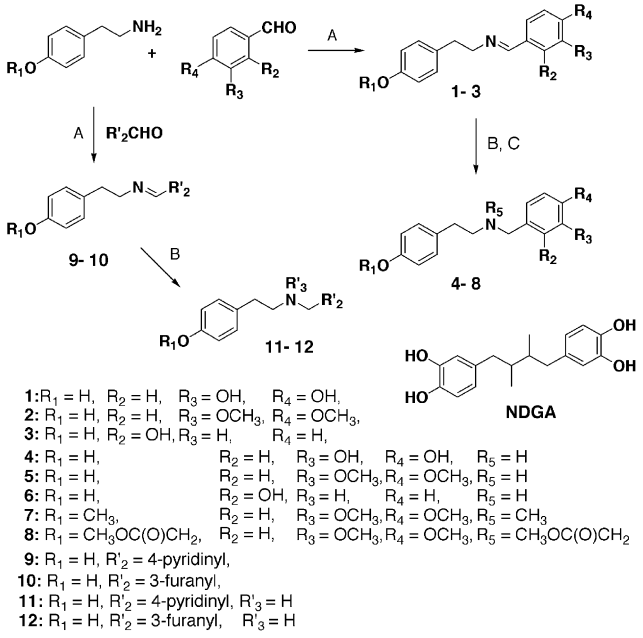

Figure 2. Syntheses of designed compounds and structure of NDGA: (A) benzene, reflux; (B) $NaBH_4$, EtOH; (C) 60% NaH, alkyl halide, DMF.

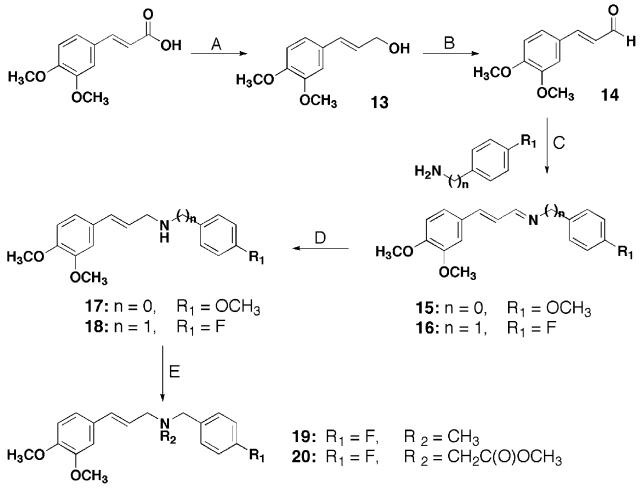

Figure 3. Syntheses of designed compounds: (A) (1) NEt_3, methyl chloroformate, −7 °C, THF, (2) $NaBH_4$, MeOH, 1 h, 10 °C, THF, (3) 1 N HCl; (B) (1) DMSO, CH_2Cl_2, oxaly chloride, −78 °C, (2) starting material, CH_2Cl_2, (3) NEt_3; (C) benzene, reflux; (D) EtOH, $NaBH_4$; (E) 60% NaH, alkyl halide, DMF.

Compounds **17** and **18** were prepared from the reaction of (*E*)-3-(3,4-dimethoxy-phenyl)propenal (**14**) with the corresponding amines, followed by reduction using $NaBH_4$ (Fig. 3). Compounds **19** and **20** were also prepared from the reaction of [3-(3,4-dimethoxyphenyl)-allyl](4-fluorobenzyl)amine (**18**) with iodomethane or methyl bromoacetate using the method described for **7**. The structures of the compounds were confirmed by ^1H NMR, ^{13}C NMR, and elemental analysis.

In NCED test a few chemicals showed inhibitory activity among the synthesized chemicals. In Table 1

Table 1. NCED inhibitory activity of synthesized compounds

	IC_{50} (μM)
NDGA	183 ± 28
6	160 ± 35
7	87 ± 14
17	145 ± 19
20	55 ± 9

NCED activity was estimated in the presence of 23 μM 9′-*cis*-neoxanthin at pH 7.0 at 20 °C. The product, C25-apocarotenoid, was analyzed by HPLC at 440 nm wavelength.

chemicals possessing NCED inhibitory activity are listed. The inhibitory activity was estimated by comparing the amount of resulted C_{25}-apocarotenoid in chemical-treated reaction medium and nontreated reaction medium. Among the chemicals compound**20** showed the most potent activity. This indicates that NDGA, compounds **6**, **7**, **17**, and **20** are NCED inhibitors and therefore can be ABA biosynthesis inhibitors in vivo.

Next, to test the efficacy of NDGA and synthesized chemicals as an ABA biosynthesis inhibitor, stomatal closure assay was examined. In guard cells, ABA regulates stomatal apertures by inhibiting stomatal opening and inducing stomatal closure in response to drought stress.[34] The ABA levels increased 10-fold more in water-deficit stressed tissues than in nonstressed tissues.[35] A high concentration of mannitol is expected to mimic the effect of water-deficit stress and increase the ABA concentration. That is, incubation in 0.4 M mannitol imposes osmotic stress on guard cells. As illustrated in Figure 4, we treated epidermal cells from spinach with 100 μM NCED inhibitors, NDGA or 10 μM ABA to assess the effect of this inhibitor on stomatal closure. Treatment with 0.4 M mannitol caused stomatal closure similar to that seen with 10 μM ABA. Under these conditions, NDGA, compounds **7**, **17**, and **20** inhibited stomatal closure. Other compounds were inactive. Compound **20** was found to be the most potent

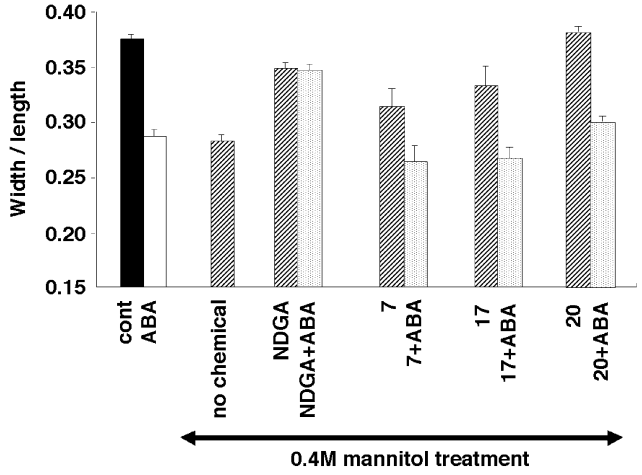

Figure 4. The effect of compounds on closing of stomatal aperture. Epidermal strips were immersed in 0.4 M mannitol with or without 100 μM inhibitors or 10 μM ABA and incubated for 3 h. The data show the mean ± SE of three independent experiments measuring at least 100 stomata. All solutions included DMSO at ca. 0.02%.

inhibitor of stomatal closure. The co-application of 10 μM ABA and 100 μM **20** produced a response similar to that with ABA alone (Fig. 4). In contrast, NDGA abolished the promotion of stomatal closure when co-applied with ABA, although it reduced stomatal closure in 0.4 M mannitol. This observation should be due to the side effects of NDGA.

The ABA content of spinach leaves was determined after incubation in 0.4 M mannitol. Spinach leaf slices were incubated for 2 h in 10 mM HEPES (pH 6.5 with KOH) with inhibitor at 100 μM. Subsequently, the leaf slices were incubated for 4 h in 10 mM HEPES with 0.4 M mannitol, and with or without inhibitor. Following incubation, the slices were homogenized and extracted in 80% methanol with 200 mg/L BHT. The ABA purified by HPLC (Shiseido CAPCELL PAK C18 column, 4.6 × 250 mm) was methylated with diazomethane. GC–MS analysis was carried out on a JEOL Auto-mass JMS-AM 150 mass spectrometer connected to a Hewlett-Packard 5890-A-II gas chromatograph with a capillary DB-1 (J&W Scientific, Folsom, CA) column (0.25 mm × 15 m, 0.25 mm film thickness). [$^{13}C_2$]-ABA was used as an internal standard.[36] The GC–SIM responses at m/z 190 (base peak of ABA) and m/z 192 (base peak of $^{13}C_2$-ABA) were monitored.

NDGA, compounds **7**, **17**, and **20** at 100 μM were examined for ABA biosynthesis inhibitory activities under osmotic stress three times. As shown in experiment 1 of Table 2, the ABA content in leaf slices treated with 0.4 M mannitol increased 7.8-fold to 22.7 ng/g FW in 10 mM HEPES (pH 6.5). Treatment with compounds **7** and **17** resulted in no significant reductions in ABA biosynthesis under osmotic stress. Nevertheless, compound **20**, which results from the replacement of the *N*-methyl group of compound **19** by *N*-acetic acid methyl ester, inhibited about 38% of ABA accumulation (14.0 ng/g FW). NDGA was less active than compound **20**. These results demonstrate that compound **20** is the strongest ABA biosynthesis inhibitor among the tested compounds. The test was repeated in triplicate under the same experimental conditions and similar results were obtained as shown in Table 2. In a previous study,[25] NDGA significantly inhibited ABA accumulation under similar conditions as used here; however, this compound did not show clear inhibitory activity in our experiments.

Table 2. The effect of compounds on ABA accumulation in spinach leaf slices

Experiment	1	2	3
	ABA (ng/g FW)	ABA (ng/g FW)	ABA (ng/g FW)
Control	2.90	11.3	17.8
0.4 M mannitol	22.7	178.4	134.9
100 μM NDGA	20.5	165.3	128.5
100 μM **7**	22.3	174.4	123.5
100 μM **17**	26.1	168.7	137.1
100 μM **20**	14.0	82.8	65.6

Spinach leaf slices were treated with each 100 μM of NDGA, compound **7**, **17**, or **20** in 0.4 M mannitol solution (10 mM HEPES buffer). FW = fresh weight.

Eventually we found that **20** should be an ABA biosynthesis inhibitor. On the basis of this study, it can be estimated that the essential structures required for NCED inhibitory activity of this chemical group should be an amine moiety, which is substituted with phenylalkyl group, phenylalkenyl group, and carboalkoxyalkyl group. As there still remains a lot of room for structural modification of this chemical group, the further study on structure–activity relationships will lead us to find more potent and specific NCED inhibitors. The result thus obtained will play an important role for designing new inhibitors. Recently 'chemical genetics' has been used as a new tool for dissecting and understanding biological systems in plants.[37,38] Development of chemicals that induce phenotypes of interest is now emerging as a useful way to study biological systems in plants and this would be a complement to classical biochemical and genetic methods. In this context ABA biosynthesis inhibitor could be a new player in this field.

Acknowledgements

We thank Mrs. M. Kobayashi and A. Hanada (RIKEN, Plant Science Center) for helpful technical assistance in the ABA measurements. This research was supported in part by the Bioarchitect Research Program at RIKEN, funded by the Science and Technology Agency of Japan.

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