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Phytotoxic effects of selected *N*-benzyl-benzoylhydroxamic acid metallo-oxygenase inhibitors: investigation into mechanism of action

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Treatment of *Arabidopsis thaliana* with 100 μM hydroxamic acids **F1** and **F2**, found previously to inhibit carotenoid cleavage dioxygenase enzyme CCD1, was found to cause chlorophyll bleaching and phytotoxicity. A further set of hydroxamic acid analogues was synthesised, and these compounds were found to be phytotoxic towards *A. thaliana* at 16–400 μM, and to show some phytoxicity towards broad-leaved weeds *C. album* and *S. media* at 100 μM. Compound **F1** was found to inhibit *p*-hydroxy-phenylpyruvate dioxygenase (HPPD), a known herbicide target (IC_{50} 30 μM), but compounds **F5** and **F8** showed no inhibition of HPPD, despite **F8** showing higher levels of phytotoxicity. Plants grown in the presence of **F1** or **F5** that were treated with 50 μM homogentisic acid showed partial recovery of growth, indicating some inhibition of HPPD *in planta*. These are the first hydroxamic acid inhibitors reported for HPPD, but the results indicate that inhibition of HPPD is only partly responsible for the observed phytotoxicity, and that another unknown metalloenzyme is also targeted by these compounds.

Introduction

The carotenoid cleavage dioxygenases (CCDs) catalyse the oxidative cleavage of a range of carotenoid substrates in plants, mammals and bacteria (see Fig. 1A), to form apocarotenoid cleavage products.[1,2] In plants, which possess multiple CCD genes, carotenoid oxidative cleavage is a key step in the biosynthesis of abscisic acid,[3] and in the biosynthesis of the recently-discovered strigolactone shoot branching hormone,[4,5] and apocarotenoid natural products such as β-ionone and geranyl-acetone plant volatiles.[6]

In order to study the function of members of the CCD family using a chemical genetics approach, we have previously developed a class of hydroxamic acid inhibitors of tomato CCD1, in which varying the aryl–N distance in the inhibitor was found to influence the specificity of enzyme inhibition.[7] Hydroxamic acids in the D and F series showed inhibition of CCD1, a 9,10-cleavage enzyme (see Fig. 1B), and some compounds also

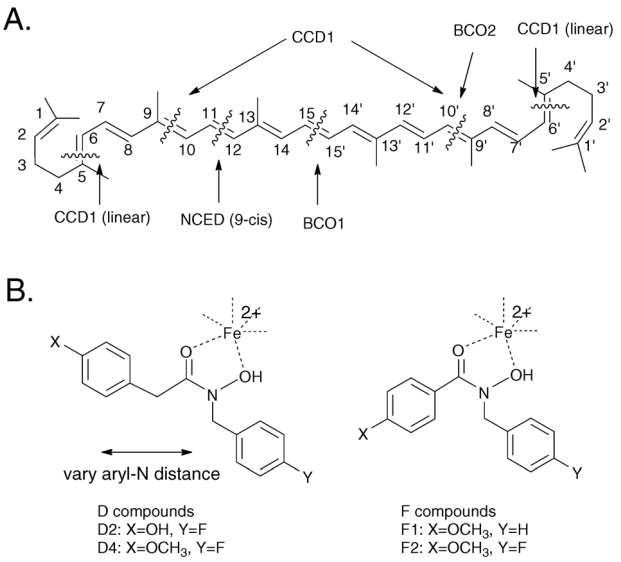

Fig. 1 (A) Carotenoid oxidative cleavage reactions catalysed by CCD enzymes; (B) design of CCD hydroxamic acid inhibitors.

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showed inhibition of 9-*cis*-epoxycarotenoid dioxygenase (NCED), an 11,12-cleavage enzyme, and some compounds also showed a shoot branching phenotype *in planta*.[7] In the course of this study, we observed that 2 out of a collection of 20 hydroxamic acids, compounds **F1** and **F2**, caused loss of chlorophyll (bleaching) from leaves of *A. thaliana* when grown on agar plates. Since new mechanisms for selective plant phytotoxicity are of potential interest for crop protection, we have investigated this observation further, and we report structure–activity data and investigation of the mechanism of action of these compounds.

Results and discussion

Phytotoxicity of F1 & F2

In our previous study, we found that hydroxamic acids in the D and F series (see Fig. 1) inhibited the 9,10 cleavage enzyme CCD1 *in vitro*.[7] Unexpectedly, compounds **F1** and **F2** caused bleaching and death of *A. thaliana* plants when incorporated at 100 μM into agar plates (see Fig. 2). None of the other compounds exhibited this property, even compounds which contained just an extra methoxy group at the 3′ position on the aryl ring. Since the D compounds showed similar or greater activity against CCD1, yet did not show the bleaching phenotype, it seems unlikely that CCD1 is the target for phytotoxic activity. Therefore, the molecular target for **F1** and **F2** may be a non-CCD oxidative enzyme with a non-heme iron or other metal ion co-factor.

Structure–activity studies

Further hydroxamic acids **F5**, **F6** and **F8** containing chlorine, methoxy and/or amino substituents on the same carbon skeleton were synthesized. The synthetic route, as described previously,[7] involves alkylation of *O*-benzyl, *N*-Boc-hydroxylamine, followed by Boc deprotection and coupling to the required substituted benzoic acid (see Fig. 3).

The new analogues were found to be phytotoxic at 16–210 μM against *A. thaliana* in agar media (see Tables 1 and 2), whereas no bleaching was observed using hydroxamic acids **D2** and **D4**, containing a phenylacetyl rather than benzoyl substituent. Compounds **F6** showed strong inhibition of CCD1, comparable with **F1** and **F2**. The hydroxamic acids and a recently commercialised HPPD-inhibiting herbicide tembotrione,[8] were tested for phytotoxicity against *A. thaliana* and broad-leaved weeds.

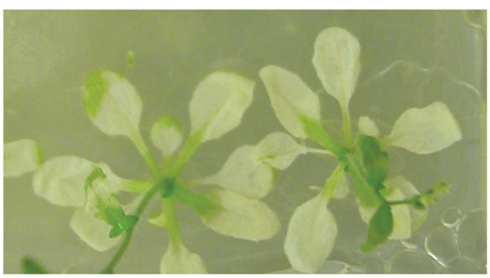


Fig. 2 *A. thaliana* grown on agar media until flower initiation in the presence of 100 μM hydroxamic acid **F1**. Bleaching was not observed in the absence of **F1**.

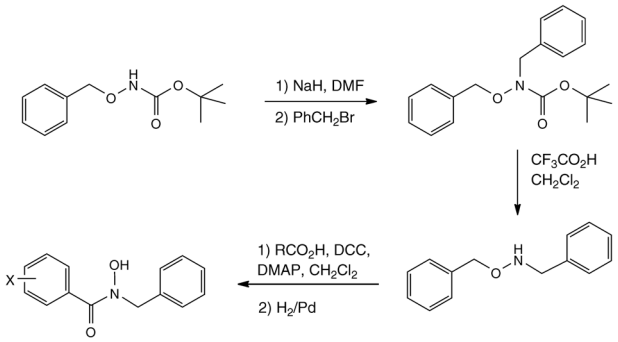


Fig. 3 Synthetic route for hydroxamic acids.

Table 1 Phytotoxicity and inhibition of *A. thaliana* HPPD and LeCCD1 by hydroxamic acids

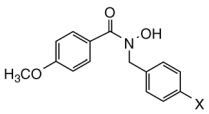
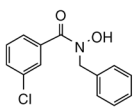
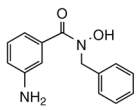
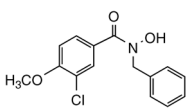
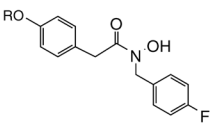
Inhibitor		Phyto-toxicity	% inhibition of *A. thaliana* HPPD @ 50 μm	% inhibition of LeCCD1 @ 100 μm
	F1 X = H	+	85	>95
	F2 X = F	+	70	>95
	F5	+	0	0
	F6	+	90	>95
	F8	+	0	30
	D2 R = H	–	90	>95
	D4 R = CH_3	–	60	>95

Table 2 LC_{50} values (in μM) for each inhibitor against *A. thaliana* and two broad-leaved weeds *Chenopodium album* and *Stellaria media*, grown in agar. No bleaching or phytotoxicity was observed with compounds **D2** and **D4**

	F1	F5	F6	F8	Tembotrione
Arabidopsis thaliana	16	38	70	210	0.012
Chenopodium album	740	310	>1000	40	0.036
Stellaria media	670	90	820	370	0.036

When applied in agar media, **F1**, **F2**, **F5**, and **F6** showed activity against *A. thaliana* at 100 μM concentration, with **F8** showing slightly lower activity (see Table 2). Compound **F8** also showed some herbicidal activity against broad-leaved weed *Chenopodium album*

at 100 μM concentration, and compound **F5** was active against *Stellaria media*, whereas compounds **F1** and **F2** were inactive against these plants. Different phenotypic effects were seen: compound **F8** stunted the growth of the *C. album* seedlings and turned them completely white; whereas compound **F5** prevented germination of *S. media* (data not shown).

Inhibition of *A. thaliana p*-hydroxyphenylpyruvate dioxygenase

p-Hydroxyphenylpyruvate dioxygenase (HPPD, see Fig. 4), a metallo-oxygenase enzyme involved in ubiquinone biosynthesis in plants, is a known herbicide target enzyme.[9] Given the structural resemblance of **F1** & **F2** to the substrate *p*-hydroxyphenylpyruvic acid, it seemed quite likely that inhibition of HPPD might be the mechanism of action of these compounds. Herbicide inhibitors of HPPD are known to act *via* chelation of the iron(II) cofactor,[9–11] which is the mechanism of inhibition of CCD enzymes by these compounds, though no hydroxamic acid inhibitors have been reported for HPPD.

Recombinant *A. thaliana* HPPD[12,13] was expressed in *Escherichia coli*, and the enzyme was purified to homogeneity using a literature protocol.[13] The HPPD reaction can be monitored by complexation of the enol form of *p*-hydroxyphenylpyruvic acid with borate, giving rise to an absorbance at 320 nm.[14] Incubation with

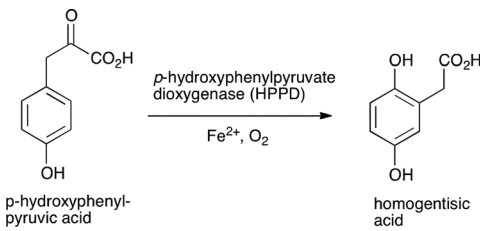


Fig. 4 Reaction catalysed by *p*-hydroxyphenylpyruvate dioxygenase (HPPD).

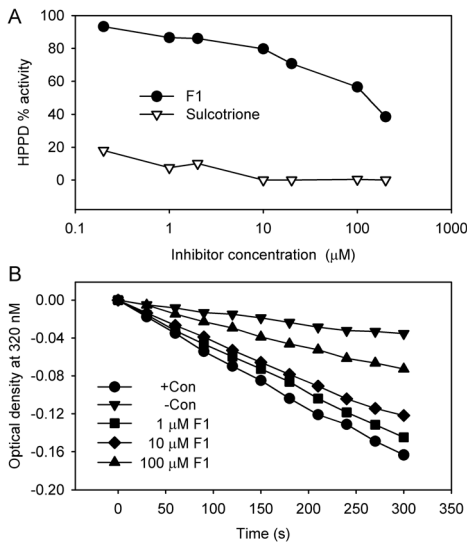


Fig. 5 Inhibition of *A. thaliana p*-hydroxyphenylpyruvate dioxygenase by hydroxamic acid **F1** using (A) HPLC assay, compared with a known HPPD inhibitor sulcotrione;[9] (B) enol-borate assay. Controls lacked inhibitor and were with (+Con) or without (−Con) addition of enzyme.

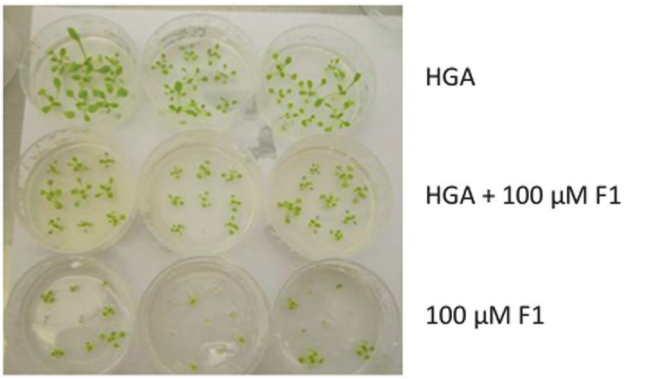


Fig. 6 Growth of *A. thaliana* in the presence of 50 μM homogentisic acid (HGA) and 100 μM hydroxamic acid **F1** for 20 days.

increasing concentrations of **F1** gave rise to enzyme inhibition using this assay method (see Fig. 5A). Enzyme inhibition was also observed using an HPLC assay method (see Fig. 5B).[14] The two methods gave somewhat different IC_{50} values of 30 μM and 100 μM respectively for compound **F1**. The level of inhibition increased upon pre-incubation with enzyme over 10–20 min (data not shown), indicating a time-dependent onset of inhibition.

Hydroxamic acids **F6** and **F7** also showed inhibition of *A. thaliana* HPPD using the enolborate method, giving 90% and 85% inhibition respectively at 50 μM concentration, however, compounds **F5** and **F8** showed no measurable inhibition of HPPD at 50 μM concentration, despite **F8** showing higher phytotoxicity (see Table 1). Compounds **D2** and **D4** prepared previously,[7] based on substituted phenylacetic acids rather than benzoic acid, also showed 90% and 60% inhibition of HPPD at 50 μM concentration, but showed no phytotoxicity phenotype.

"Metabolite rescue" experiment

Given the lack of correlation between HPPD inhibition and phytotoxicity, a further experiment was carried out in which *A. thaliana* plants grown in presence of 100 μM **F1** or **F5** were treated with homogentisic acid (HGA), the product of the HPPD-catalysed reaction. If HPPD inhibition was occurring in the plant, then addition of HGA should restore the ability to biosynthesise ubiquinone, and hence restore growth. As shown in Fig. 6, treatment with HGA was found to partially alleviate the toxicity caused by **F1**; mean plant fresh weight with **F1** alone (1.8 ± 0.6 mg) was increased to 3.3 ± 0.3 mg by the **F1** + HGA treatment ($P < 0.05$, n = three plates, 9 seeds per plate), suggesting that some inhibition of HPPD is taking place in the plant. Similar results were obtained with compound **F5**.

Conclusions

We have observed a selective phytotoxicity phenotype in a small group of hydroxamic acids based upon substituted benzoic acid skeletons. Compounds **F1** and **F2**, as well as analogue **F6**, inhibit the known herbicide target enzyme HPPD with IC_{50} values of <50 μM, and are the first hydroxamic acid inhibitors reported for HPPD. Triketone herbicide inhibitors of HPPD

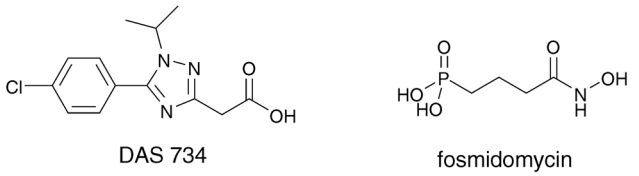


Fig. 7 Structures of metalloenzyme inhibitors that show herbicide activity.

have been shown to bind to the active Fe(II) form of the enzyme.[10,11] The time-dependent inhibition observed with these hydroxamic acids might be due to oxidation to a more tightly-bound Fe(III) form of the enzyme after ligand binding.

Surprisingly, analogues **F5** and **F8**, which show the phytotoxicity phenotype, do not inhibit HPPD. Furthermore, phenylacetyl analogues **D2** and **D4**, which do inhibit HPPD, are not phytotoxic. Hence there is not a strong correlation between HPPD inhibition and plant phytotoxicity, and these structure–activity studies raise doubts about whether HPPD is the primary target enzyme for the observed phytotoxicity.

A metabolite rescue experiment using 50 μM homogentisic acid showed partial recovery of growth, implying that some inhibition of HPPD is occurring in the plant. Therefore our conclusion is that these compounds do inhibit HPPD in the plant, but that there must be a second unknown metalloenzyme whose inhibition also leads to the observed phytotoxicity phenotype.

Other metalloenzyme herbicide targets have been described in recent years: glutamine phosphoribosyl pyrophosphoryl amidotransferase, involved in adenine biosynthesis, is the target for bleaching herbicide DAS 734 (see Fig. 7);[15] 1-deoxyxylulose 5-phosphate reductoisomerase is inhibited by the hydroxamic acid fosmidomycin (see Fig. 7);[16] and imidazole glycerol-phosphate dehydrogenase is involved in histidine biosynthesis.[17] It is therefore possible that one of these enzymes is the alternative enzyme target for phytotoxicity of these compounds.

Experimental section

Synthesis of hydroxamic acid compounds

Hydroxamic acids **F1**, **F2**, **F5**, **F6**, and **F8** were synthesised using the synthetic route shown in Fig. 3, previously described in ref. 7, from starting materials 3-chlorobenzoic acid (**F5**), 3-nitrobenzoic acid (**F6**) and 3-chloro-4-methoxybenzoic acid (**F8**). Compounds **F1** and **F2** were described in ref. 7. Data for compounds **F5**, **F6** and **F8**:

F5. δ_H (400 MHz, $CDCl_3$) 7.51 (1H, s), 7.50 (1H, d, J = 8 Hz), 7.40 (1H, d, J = 8 Hz), 7.36 (1H, t, J = 8 Hz), 7.29 (5H, m), 4.8 (2H, s); δ_C (75 MHz, $CDCl_3$) 172.9, 134.8, 130.8, 130.4, 128.5, 128.4, 127.9, 127.8, 127.1, 126.6, 125.8, 53.75; HRMS obs 263.0661, calc 263.0663 for $C_{14}H_{13}ClNO_2H^+$.

F6. δ_H (300 MHz, $CDCl_3$) 7.35 (5H, m), 7.28 (1H, d, J = 8 Hz), 7.15 (1H, t, J = 8 Hz), 6.86 (1H, s), 6.77 (1H, d, J = 8 Hz), 4.84 (2H, s); δ_C (75 MHz, $CDCl_3$) 172.1, 142.9, 129.0, 128.7, 128.6, 128.5, 128.5, 128.4, 128.0, 116.2, 113.8, 53.8; HRMS obs 243.1131, calc 243.1128 for $C_{14}H_{15}N_2O_2H^+$.

F8. δ_H (300 MHz, d_6-DMSO) 7.8 (1H, s), 7.71 (1H, d, J = 8 Hz), 7.34 (5H, m), 6.95 (1H, d, J = 8 Hz), 4.83 (2H, s), 3.90 (3H, s); δ_C (75 MHz, d_6-DMSO) 156.0, 131.0, 130.8, 129.8, 128.8, 128.7, 128.5, 128.0, 120.0, 113.7, 56.7, 53.2, carbonyl not seen; HRMS obs 292.0739, calc 292.0735 for $C_{15}H_{14}ClNO_3H^+$.

Enzyme inhibition assays

A. thaliana p-hydroxyphenylpyruvate dioxygenase was overexpressed and purified as previously described.[11] The enol-borate assay was performed essentially as described.[10] Briefly, 4-hydroxyphenylpyruvic acid (pHPP, Sigma) was prepared in keto form by dissolving in ethanol at 100 mM and then diluting to 10 mM with 0.2 M Tris·HCl pH 7.5. A solution of 1.5 M sodium ascorbate, 10 mM $FeSO_4$, 25 mg ml^{-1} catalase (Sigma C9322, 2000–5000 units mg^{-1}) and 10 mM pHPP was then prepared and then diluted 100-fold with 0.8 M K_2HPO_4, 0.4 M H_3BO_3, 0.2 M Tris·HCl pH 7.5. This reaction mixture was allowed to equilibrate at room temperature for 10 min to form the enol-borate complex. To initiate the reaction, 0.9 ml of this reaction mix was added to 100 μl of crude lysate from the HPPD-expressing *E. coli* strain (containing 10 mM HEPES pH 7.0 and 1 mM $FeSO_4$). The O.D. at 320 nm was then followed for 5 minutes. The HPLC assay for HPPD activity was performed as previously reported[12] except that purification of the enzyme reactions was by precipitation in the presence of 50% methanol followed by centrifugation at 20 000 g for 10 min; supernatant was used for HPLC analysis.

Phytotoxicity testing

Plant agar-based growth media and environmental growth conditions for *Arabidopsis* and for weed species were as described previously.[7] Agar media were supplemented with the indicated inhibitors and/or HGA. Weed seeds were obtained from Herbiseed, Berkshire, UK.

Acknowledgements

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