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Non-natural cinnamic acid derivatives as substrates of cinnamate 4-hydroxylase

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Abstract

Cinnamate 4-hydroxylase (C4H), a monooxygenase in the plant phenylpropanoid pathway, was assayed for its ability to hydroxylate 29 substrate analogues. Nine of the tested analogues with various aromatic side chains, including 3-coumaric acid, were metabolized by C4H. Seven products from these reactive analogues were characterized using LC/MS, ^{1}H NMR and ^{13}C NMR spectroscopic analysis. For example, caffeic acid was the product of 3-coumaric acid. The products 4-hydroxy-2-chlorocinnamic acid and 4-hydroxy-2-ethoxy-cinnamic acid are novel compounds that have not been previously reported. The kinetic parameters of C4H towards these analogues were determined.

Keywords: *Arabidopsis thaliana*; Cruciferae; *Saccharomyces cerevisiae*; Cytochrome P450; Phenylpropanoids; Coumaric acid

1. Introduction

Cinnamate 4-hydroxylase (C4H; EC 1.14.13.11) is a cytochrome P450 in the plant phenylpropanoid pathway, which leads to many important secondary metabolites such as lignins, coumarins, and flavonoids (Humphreys and Chapple, 2002; Weisshaar and Jenkins, 1998). These compounds are essential to form structural components, pigments, antioxidants, signaling molecules, UV-protective compounds, antibiotics and anti-insect compounds in plants (Morant et al., 2003; Taylor and Grotewold, 2005; Weisshaar and Jenkins, 1998). Together with the NADPH-P450 reductase and the cofactor NADPH, C4H catalyzes the *para*-hydroxylation of *trans*-cinnamic acid **1** to *p*-coumarate **2** (Fig. 1).

In addition to its natural substrate, C4H has the ability to metabolize some substrate analogues, including xenobiotics (Schalk et al., 1997a,b; Urban et al., 1994). These analogues are derivatives of cinnamate (**1**) with a planar structure, negatively charged side chain and a size of less than two aromatic rings (Schoch et al., 2003). The metabolism of unnatural substrates by C4H provides the possibility to synthesize novel products from phenylpropanoid pathway, if other enzymes in this pathway can further metabolize those products. Also, since there is no crystal structure currently available for C4H, the investigation of alternative substrates provides insight into the binding and reaction mechanism of C4H (Schoch et al., 2003).

In this study, we tested the ability of recombinant C4H from *Arabidopsis thaliana* (CYP73A5) towards 29 analogues of *trans*-cinnamic acid (**1**). Several analogues are hydroxylated by C4H, and we characterized the structures of the products and the kinetic parameters of the reactions.

2. Results and discussion

2.1. Alternative substrates of C4H

Based on structural similarity to cinnamate **1**, we first tested the reactivity of 15 analogues of *trans*-cinnamic acid **1** with different side chains on the aromatic ring in place of acrylic acid, e.g., eugenol and phenylacetic acid, but none

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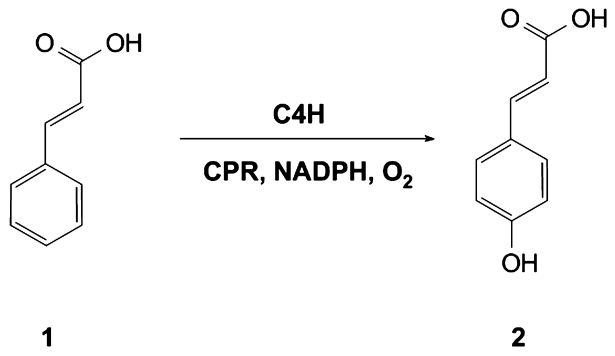
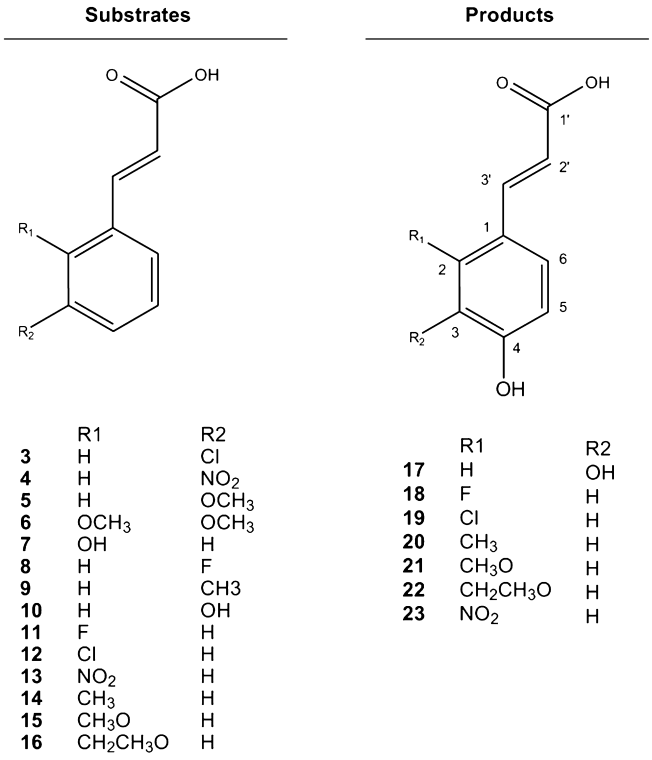


Fig. 1. Reaction catalyzed by C4H in plants.

disappeared from solution in the in vivo feeding study with recombinant C4H (data not shown). Next we similarly tested the reactivity of 14 analogues with substitution at R1 or R2 or both positions (Fig. 2). Compounds **3**–**7** were not metabolized by the WAT11 host expressing C4H enzyme. However, we can not rule out the possibility that the absence of reactivity could be due to lack of uptake of specific analogues. All analogues that were hydroxylated are R1-substituents except 3-fluorocinnamic acid **8**, 3-methylcinnamic acid **9**, and 3-hydroxycinnamic acid **10**. No formation of products was observed from analogues fed to the control yeast strain lacking C4H.



	R1	R2
3	H	Cl
4	H	NO_2
5	H	OCH_3
6	OCH_3	OCH_3
7	OH	H
8	H	F
9	H	CH3
10	H	OH
11	F	H
12	Cl	H
13	NO_2	H
14	CH_3	H
15	CH_3O	H
16	CH_2CH_3O	H

	R1	R2
17	H	OH
18	F	H
19	Cl	H
20	CH_3	H
21	CH_3O	H
22	CH_2CH_3O	H
23	NO_2	H

Fig. 2. Substrate analogues and products of C4H. Substrate analogues (1 mM) were incubated at 30 °C for 2 h with 10 mL of induced WAT11 cells expressing C4H activity. Substrates **3**–**9** were identified as non-reactive if no decrease in concentration from HPLC chromatograms was observed.

This result shows that the substrate recognition sites of C4H accept many cinnamic acid analogues with R1-substitutions of various size and polarity. This finding is consistent with previous reports of C4H activity against naphthalene derivatives, whose R1-substituted analogues were similar in size (Pierrel et al., 1994; Schalk et al., 1997a,b). However, compounds **8**–**10** with substitutions at R2 were also metabolized. In general, steric effects and hydrophobic interactions are keys to the specificity of P450s (De Voss et al., 1997). It is relatively difficult to deduce any influence on reactivity caused by the electronic character of the analogues with substitution on R1, since both electron withdrawing (F, Cl, NO_2) and electron donating (methyl, methoxy, ethoxy) substitutions were hydroxylated.

2.2. Structures of products from substrate analogues

From a comparison of HPLC retention time, LC/MS molecular mass and UV-spectra between the product derived from 3-hydroxycinnamic acid **10** and a caffeic acid **17** standard, we confirmed the product is caffeic acid **17**. The LC/MS results for the products from compounds **8**–**16** are consistent with the predicted molecular weights of the hydroxylated substrate analogues.

The ^{1}H NMR and ^{13}C NMR data, UV spectra, and HR-ESI-MS data for the metabolized products of substrates **10**–**15** are listed in Tables 1 and 2, respectively. Based on the analysis of proton couplings, we propose that the new hydroxyl group is always at the 4-position. The structures of compounds **18**–**22** are 2-fluoro-4-hydroxycinnamic acid, 2-chloro-4-hydroxycinnamic acid, 2-methyl-4-hydroxycinnamic acid, 2-methoxy-4-hydroxycinnamic acid, and 2-ethoxy-4-hydroxycinnamic acid, respectively (Fig. 2). Based on the analysis of ^{1}H, ^{13}C, ^{1}H–^{1}H COSY, ^{1}H–^{13}C HMQC, and ^{1}H–^{13}C HMBC spectra, we assigned the positions of protons and carbons for **18**–**22** (Tables 1 and 2). The structure of compound **23** is proposed as 2-nitro-4-hydroxycinnamic acid (Tables 1 and 2). Based on the analysis of long range coupling of ^{1}H and ^{13}C, the hydroxyl group in compound **23** is likely *para*- to the acrylic acid side chain. Due to the low activity of C4H against substrates **8** and **9**, the products from these two analogues were not available in sufficient quantity for NMR experiments.

To the best of our knowledge, this is the first time these analogues have been shown to be metabolized by C4H. The products 4-hydroxy-2-chlorocinnamic acid **19** and 4-hydroxy-2-ethoxycinnamic acid **22** are novel compounds that have not been previously reported. The products **18**, **20**, **21**, and **23** have been reported as intermediates for fine chemicals syntheses in patents or the chemical literature (Kaku et al., 2004; Bloom, 1959; Oonuma et al., 1993; Khanna, 1983). Compound **21** was tentatively identified in *Hemarthria altissima* (Tang and Young, 1982). Caffeic acid **17**, the product of compound **10**, is an antioxidant found in various plants (Yamanaka et al., 1997).

Table 1
^{1}H (500 MHz, CD3OD) NMR data [δ (ppm), m, J (Hz)][a], UV spectra [λ_{max} (MeOH), nm], and HR-ESI-MS data for the products of C4H substrate analogues

Substitution	F	Cl	CH_3	OCH_3	OCH_2CH_3	NO_2
Position	**18**	**19**	**20**	**21**	**22**	**23**
3	6.51 *dd* (12.5, 2.5)	6.82 *d* (2.5)	6.57	6.40 *d* (2.0)	6.38 *d* (2.5)	7.31 *d* (2.5)
5	6.61 *dd* (8.5, 2.5)	6.72 *dd* (8.5, 2.5)	6.56	6.36 *dd* (8.5, 2.0)	6.35 *dd* (8.5, 2.5)	7.07 *dd* (8.5, 2.5)
6	7.46 *t* (8.5)	7.58 *d* (8.5)	7.40 *d* (9.0)	7.36 *d* (8.5)	7.35 *d* (8.5)	7.66 *d* (8.5)
2′	6.34 *d* (16.0)	6.29 *d* (16.0)	6.24 *d* (15.5)	6.30 *d* (16.0)	6.33 *d* (16.0)	6.32 *d* (16.0)
3′	7.66 *d* (16.0)	7.96 *d* (16.0)	7.77 *d* (15.5)	7.84 *d* (16.0)	7.84 *d* (16.0)	7.87 *d* (16.0)
R1			2.28 *s*	3.81 *s*	4.02 *q* (7.0) 1.42 *t* (7.0)	
λ_{max}(nm)	289, 305	296	315	287, 323	284, 321	256, 289
HR-ESI-MS *m*/*z*	182.0392	198.0094	178.0634	194.0583	208.0752	209.0334
Calc.[b]	182.0379	198.0084	178.0630	194.0579	208.0736	209.0324

[a] Assignments were based on COSY, HMQC, and HMBC experiments.
[b] Calculated for the formula $C_9H_7O_3R$, R = substitutions.

Table 2
^{13}C (125 MHz, CD3OD) NMR data of the products from C4H reaction with analogues[a]

Position	**18**[b]	**19**	**20**	**21**	**22**	**23**
1	114.8 *d* (12)	124.7	124.5	114.6	116.1	121.6
2	163.8 *d* (273)	136.9	139.6	160.2	160.9	151.2
3	103.9 *d* (24)	116.2	116.8	98.4	100.7	112.0
4	162.8 *d* (10)	161.6	159.3	161.3	162.6	161.0
5	113.3	117.4	113.3	107.6	109.0	121.6
6	131.2 *d* (5)	130.0	127.8	129.9	131.5	131.2
1′	172.0	170.5	170.4	170.3	171.8	170.4
2′	118.3	118.4	115.6	114.0	115.4	122.0
3′	138.9	141.7	142.0	140.7	142.4	140.5
R1			18.4	54.5	65.1 15.0	

[a] Assignments were based on COSY, HSQC, and HMBC experiments.
[b] Carbons 1, 2, 3, 4, and 6 of the compound **18** are split by ^{19}F. Numbers in the brackets indicate the values of J_{CF} (Hz).

2.3. Determination of kinetic parameters

The kinetic parameters of C4H towards these analogues were determined through in vitro enzyme assays and the results are reported in Table 3. Both the K_m and the turnover number of C4H towards cinnamic acid **1** were found to be consistent with the values reported in the literature, varying from 0.7 μM to 8.9 μM, and 28 min^{-1} to 129.1 min^{-1}, respectively (Gravot et al., 2004; Hubner et al., 2003; Humphreys and Chapple, 2004; Pierrel et al., 1994; Urban et al., 1997, 1994).

Table 3
Kinetic parameters of C4H with substrate analogues

	R1	R2	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})
1	H	H	0.5 ± 0.1	102.9 ± 4.9	197.9
11	F	H	2.3 ± 0.3	164.6 ± 15.1	70.3
12	Cl	H	2.2 ± 0.4	65.8 ± 8.6	29.5
10	H	OH	13.1 ± 2.3	232.0 ± 24.2	17.8
14	CH_3	H	10.0 ± 2.4	153.4 ±2.1	15.3
16	CH_3CH_2O	H	6.5 ± 1.7	70.2 ± 15.8	10.8
15	CH_3O	H	18.4 ± 1.4	62.1 ± 34.2	3.4
13	NO_2	H	21.5 ± 2.4	27.5 ± 7.1	1.3

A reaction mixture containing 5–30 μL C4H microsomes, 0.5–40 μM substrate, and 1 mM NADPH, in a final volume of 500 μL was incubated at 30 °C for 3–10 min. Further details are described in Section 4.

Not surprisingly, C4H has the highest catalytic efficiency (k_{cat}/K_m) for the metabolism of *trans*-cinnamic acid **1**. The K_m values for 2-fluorocinnamic acid **10**, 2-chlorocinnamic acid **11** and 2-ethoxycinnamic acid **15** are also close to that of K_m for **1**. Of all the analogues, the metabolism of **10** by C4H has the highest efficiency (70.3 min^{-1} μM^{-1}) and the second highest turnover number (164.6 ± 15.1 min^{-1}). The K_m and catalytic efficiency for the metabolism of **10** and **11** were comparable to another reported substrate analogue of C4H, 2-naphthoic acid (Schalk et al., 1997a).

3. Concluding remarks

Most previous reports of active C4H substrate analogues are mimics of 2-naphthoic acid with two heterocyclic rings (Pierrel et al., 1994; Schalk et al., 1997a,b). With the exception of 2-naphthoic acid, C4H had a very low catalytic efficiency (less than 0.8 min^{-1} μM^{-1}) towards the other documented analogues (Pierrel et al., 1994; Schalk et al., 1997a,b). Also, with side chains other than acrylic acid, the *p*-hydroxylase function of C4H towards those reported analogues was not well maintained (Pierrel et al., 1994). Similarly, from our study, C4H did not react with analogues without acrylic acid substitution. In general, the catalytic efficiency of C4H towards R1 substituents decreased when the size of substituents increased. However, it is difficult to explain the variation of kinetic parameters because the trend does not directly correlate either with the size or the electronic effects of the analogues.

4. Experimental

4.1. General

4.1.1. Chemicals

Isoeugenol and 2-chlorocinnamic acid were purchased from ACROS Organics (Geel, Belgium). 3-Methoxycinnamic acid, 2-methyl cinnamic acid, eugenol, 2-methoxycin-

namic acid, and 4-hydroxy-3-methoxy phenyl acetone were purchased from Alfa Aesar (Ward Hill, MA). Benzene and toluene were from Mallinckrodt Baker, Inc. (Paris, Kentucky). 4-Hydroxy-3-methoxyphenyl pyruvic acid was purchased from TCI America (Portland, OR). Yeast nitrogen base without amino acids and ammonium sulfate (YNB-AA/AS) was purchased from Becton Dickinson (Sparks, MD). 3-Nitrocinnamic acid, cinnamyl acetate, methyl *trans*-cinnamate, 2-fluorocinnamic acid, *trans*-2,3-dimethoxycinnamic acid, 3-fluorocinnamic acid, *trans*-2-methoxy-4-(2-nitrovinyl) phenol, 3-methylcinnamic acid, benzoic acid, 2-ethoxycinnamic acid, 3-cholorocinnamic acid, homovanillic acid, 2-hydroxy cinnamic acid, 3-hydroxycinnamic acid, 2-nitrocinnamic acid, phenylacetic acid, tropic acid, mandelic acid, hippuric acid, glucose, galactose, casein enzymatic hydrolysate (EHC), L-tryptophan, methanol, acetonitrile, dimethyl sulfoxide (DMSO), *trans*-cinnamic acid and *p*-coumaric acid were of the highest purity and purchased from Sigma–Aldrich Co. (St. Louis, MO).

4.1.2. HPLC method

Analysis of the in vitro reaction was performed by HPLC using an Agilent (Palo Alto, CA) SB-C18 column (4.6 × 75 mm) at 30 °C with a gradient elution method. The flow rate was 0.9 mL/min and the injection volume was 10 μL. Solvent A was 1.5% (v/v) AcOH in H_2O and solvent B was CH_3CN. Initially, solvent B was maintained at 5% for 4 min, increased to 45% over 8.5 min, and held for 1 min before returning to 5% solvent B. The UV absorbance at λ_{max} (Table 2) for respective product was recorded.

4.1.3. Mass spectrometry

Electrospray ionization (ESI) analyses were carried out on a Waters Micromass Q-TOF system (Waters, Milford, MA) after HPLC on a Waters C18 column (2.1 × 150 mm) with a flow rate of 0.2 mL/min by a gradient elution method. Initially, solvent B was maintained at 5% for 8 min, increased to 45% over 17 min, and held for 5 min before returning to 5% solvent B. The mass spectrometer scan range was from 50 to 600 *m*/*z* and detection was in the negative ion mode. Flow injection analyses were carried out on an Agilent MSD TOF mass spectrometer with an ESI source. The flow rate was 0.5 mL/min with an eluent H_2O–CH_3CN (1:1). The acquisition range was from 40 to 1000 *m*/*z* and detection was in the negative ion mode. The products from compounds **8** and **9** were analyzed with the same MS systems after HPLC on the Agilent system with the same gradient elution profile as previously described. The flow rate was adjusted to 0.8 mL/min, and the sample injection volume was 15 μl. The content of solvent A was changed to 0.1% AcOH in H_2O and solvent B was CH_3CN with 0.1% AcOH. The spectra were collected in negative ion mode.

4.1.4. NMR analyses

^1H and ^{13}C NMR spectroscopic data were obtained on Bruker DRX 500 NMR spectrometer (500 MHz) with deuterated methanol (CD_3OD) as the solvent and tetramethylsilane (TMS) as an internal standard. NMR data were reported with δ (ppm) values, and referenced to the solvent. UV spectra were obtained on a DU® Series 500 spectrophotometer (Beckman Instruments, Inc., CA) in MeOH.

4.2. Yeast strain, culture medium and growth conditions

4.2.1. Strain and plasmids

A *Saccharomyces cerevisiae* strain, WAT11, derived from the W303-B strain (*MAT a*; *ade*2-1; *his*3-11, -15; *leu*2-3, -112; *ura*3-1; *can*^{R}; *cyr*$^+$) with an *Arabidopsis thaliana* NADPH-cytochrome P450 reductase gene integrated into chromosome was used as the host strain (Pompon et al., 1996). The *S. cerevisiae* expression vector pYeDP60 driven by a *GAL10-CYC1* hybrid promoter was constructed by Pompon et al. (1996). The pYeDP60 vector containing a C4H gene from *Arabidopsis thaliana* (CYP73A5) was constructed by Humphreys and Chapple (2004). The WAT11 containing pYeDP60-C4H vector was used as host strain and the WAT11 containing an empty pYeDP60 vector was used as control strain to test for biotransformation of the substrates by the yeast host strain.

4.2.2. Culture media

The medium composition was based on a previously optimized medium for ferulate 5-hydroxylase expression in the WAT11 strain (modified synthetic medium SGI) (Jiang and Morgan, 2004). The composition of the growth medium is 20 g/L glucose, 5 g/L casein enzyme hydrolysate, 40 mg/L L-tryptophan, and 3.4 g/L YNB-AA/AS. The induction medium was optimized for C4H expression (Chen and Morgan, 2006) and the composition is 3.3 g/L glucose, 16.7 g/L galactose, 7.4 g/L casein enzyme hydrolysate, 40 mg/L L-tryptophan and 3.4 g/L YNB-AA/AS.

4.2.3. Cell cultivation procedures

A single yeast colony from a plate containing modified SGI medium was transferred to 25 mL of modified SGI medium in a 125 mL flask and cultivated at 30 °C with shaking at 300 rpm for 24–28 h until the OD_{600} reached 1.0–2.0. The cells were then subcultured to a 1 L flask with 250 mL induction medium and diluted to an initial OD_{600} of 0.02. The cell culture was induced at 30 °C for 20 h with a shaking speed of 300 rpm.

4.3. In vivo reaction for analogue screening and product preparation

For the substrate analogue activity assays, 1 mM analogue was added to 10 mL of induced cells expressing C4H at 30 °C for 2 h. Samples (500 μL) of the reaction mixture were removed and mixed with and equal volume of MeOH immediately to terminate the reaction. The mixture was centrifuged at 18,000*g* and the supernatant was analyzed by HPLC. A decrease of substrate and appearance of a new peak with earlier retention time were evidence of

reaction. For preparation of product for structural identification, substrate analogues were added at a final concentration of 1 mM–250 mL of induced yeast cells. The reactions was terminated after 26–33 h.

4.4. Extraction and purification

After reaction, the cell culture was centrifuged at 5000 rpm for 10 min at 4 °C. The supernatant was acidified by AcOH (5:1 v/v) and the mixture was extracted twice with equal volumes of EtOAc. The solvent was removed in vacuo and the sample was injected multiple times on an Agilent SB-C18 column (9.4 × 100 mm). The new product peaks were separated by an isocratic elution method. Solvent A was 1.5% (v/v) AcOH in H_2O and solvent B was 100% CH_3CN. Solvents A and B were mixed as 15:85 (v/v) at a flow rate of 4 mL/min. Product peaks were collected and the solvent was evaporated to yield dry product.

4.5. Microsome preparation and C4H quantification

After induction, microsomes were prepared essentially as described by Urban et al. (1994). The yeast cells were washed and resuspended in a high-salt Tris buffer (pH 7.6, 50 mM Tris–HCl, 20% glycerol, 4 mM EDTA and 150 mM M NaCl). Zirconia beads were added to the resuspended cells and the mixture was vortexed vigorously. After centrifugation, the pellets were discarded and PEG 3350 was slowly added to the supernatant to a concentration of 0.1 g/mL, with stirring at 4 °C for 15 min before it was centrifuged at 10,000*g* for 10 min. The pellets were resuspended in PIPES buffer (pH 7.0, 50 mM PIPES, 20% glycerol, 4 mM EDTA) using a Dounce homogenizer to create a uniform microsome suspension. Samples were divided into 500 μL aliquots and immediately stored at −80 °C.

The amount of C4H was quantified by recording the carbon monoxide difference spectra with a Varian Cary 300 dual beam spectrophotometer (Varian Inc., Palo Alto, CA) using an extinction coefficient of 91 cm^{-1} mM^{-1} (Omura and Sato, 1964). Protein concentration was quantified with the BCA™ protein assay kit (Pierce, Rockford, IL) using BSA as a standard.

4.6. In vitro enzyme kinetics assay

A series of concentrations of substrates from 0.5 μM to 40 μM were incubated at 30 °C with 1 mM NADPH. Reactions were initiated with aliquots of yeast microsomes (5–30 μL) in a total volume of 500 μL. Samples (150 μL) of the reaction mixture were taken at different time intervals and mixed with 30 μL acetic acid immediately to terminate reaction. The mixture was centrifuged at 14,000 rpm and the supernatant was analyzed by HPLC. Substrate disappearance was correlated with product peak area to achieve a standard curve of product concentration. The V_{max} was calculated from the Lineweaver–Burk plot, and the K_m was from a linear plot of reaction velocity against substrate concentration.

4.7. Identification of enzyme reaction products

Compound characterization data for products **18**–**23** are described in Tables 1 and 2 (^1H, ^{13}C and HRMS data).

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