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Benign and straightforward synthesis of lignan-type dimers using crude peroxidase from red radish (*Raphanus sativus* var sativus)

Síntesis benigna y sencilla de dímeros de tipo lignano usando peroxidasa cruda de rábano rojo (*Raphanus sativus* var sativus)

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Abstract

Alkyl esters of hydroxycinnamic acids and vanillin derivatives were used as model compounds in the oxidative coupling reactions catalyzed by a crude enzymatic extract of *Raphanus sativus* var sativus (red radish). Six products were isolated and characterized: three alkyl esters of hydroxycinnamic acids dimers and three vanilloids dimers. Herein, we report the enzymatic extract of *Raphanus sativus* var. sativus showed peroxidase activity catalyzing the oxidative coupling of phenols. Vanilloid dimers were coupled in an *ortho-ortho* way as expected, while alkyl ester dimers were formed through 5-8 and 8-8 bonds via a radical mechanism prior to cyclization.

Keywords: new enzyme sources, radish peroxidase, enzymatic oxidative coupling, phenolic dimers.

Resumen

Alquilésteres derivados de ácidos hidroxicinámicos y derivados de la vainillina fueron empleados como compuestos de referencia en reacciones de acoplamiento oxidativo. Las reacciones fueron catalizadas con extractos crudos de *Raphanus sativus* var sativus (rábano rojo). Seis productos de dimerización fueron aislados y caracterizados: tres de alquilésteres derivados de ácidos hidroxicinámicos y tres dímeros de vainilloides. En este documento se reporta que el extracto enzimático de *Raphanus sativus* var sativus presenta actividad peroxidasa ya que cataliza el acoplamiento oxidativo de distintos derivados fenólicos. Los dímeros de vainilloides se acoplaron de manera *orto-orto*, como era esperado, mientras que los dímeros de los ésteres se formaron a través de formación de enlaces 5-8 y 8-8 vía mecanismos radicalarios antes de la ciclización.

Palabras clave: nuevas fuentes de enzimas, peroxidasa de rábano, acoplamiento oxidativo enzimático, dímeros fenólicos.

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1 Introduction

Phenolic acids are secondary plant metabolites found mainly in terrestrial species. These phenolic compounds are comprised of the cinnamic C_6-C_3 family, the hydroxycinnamic acids, and their derivatives (Figure 1) with well-known bioactivity as antioxidants, free radical scavengers, anti-inflammatory and anti-microbial agents (Figueroa-Espinoza *et al*., 2005; Kundu, 2017). Their biological activity can be improved through dimerization of the phenolic monomers. Vanillin dimers have been reported to present increased antioxidant activity when compared to their monomers (de Vasconcelos *et al*., 2019). For instance, phenolic dimers are an integral part of human diet, mainly as antioxidants (Silva *et al*., 2000; Antoniotti *et al*., 2004; Moussouni *et al*., 2011; Saliu *et al*., 2011; Grúz *et al*., 2015).

Peroxidases catalyzed chemo and regioselective redox reactions over either carbon atoms or atoms with free pair electrons (Flohé, L, 2020). These enzymes could be considered as supramolecular units, and particularly the active site of peroxidases presents an iron atom that is coordinated to the porphyrin ring through four nitrogen atoms. As a result, these enzymes offer an easy way to dimerize phenolic units using H_2O_2 or peracids as an oxidative agent under mild conditions (Guo *et al*., 1997; Hamid & Khalil-ur-Rheman, 2009; Lopes *et al*., 2014; Quideau *et al*., 2014; Palade *et al*., 2019). Additionally, the use of peroxidases can lead to precipitation of phenolic compounds which facilitates their removal from aqueous solutions (Yu *et al*., 1994).

In a recent study it has been reported that the application of crude peroxidase from *Brassica oleracea* var. *alboglabra* catalyzes the oxidative coupling of guaiacol (Anita *et al*., 2014). In other work, crude peroxidase from *Brassica oleracea* var. *maraton* was investigated in the dimerization reactions of phenols (Duarte-Vázquez *et al*., 2007). In addition, peroxidase isolated from the *Momordica charantia* fruit was used to oxidize sinapic acid, affording dimers and tetramers as the main products (Liu *et al*., 2007). The four main hydroxycinnamic acids were evaluated as model substrates for the anionic potato peroxidase (Arrieta-Báez & Stark, 2006).

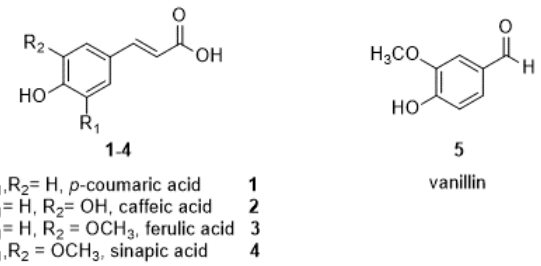
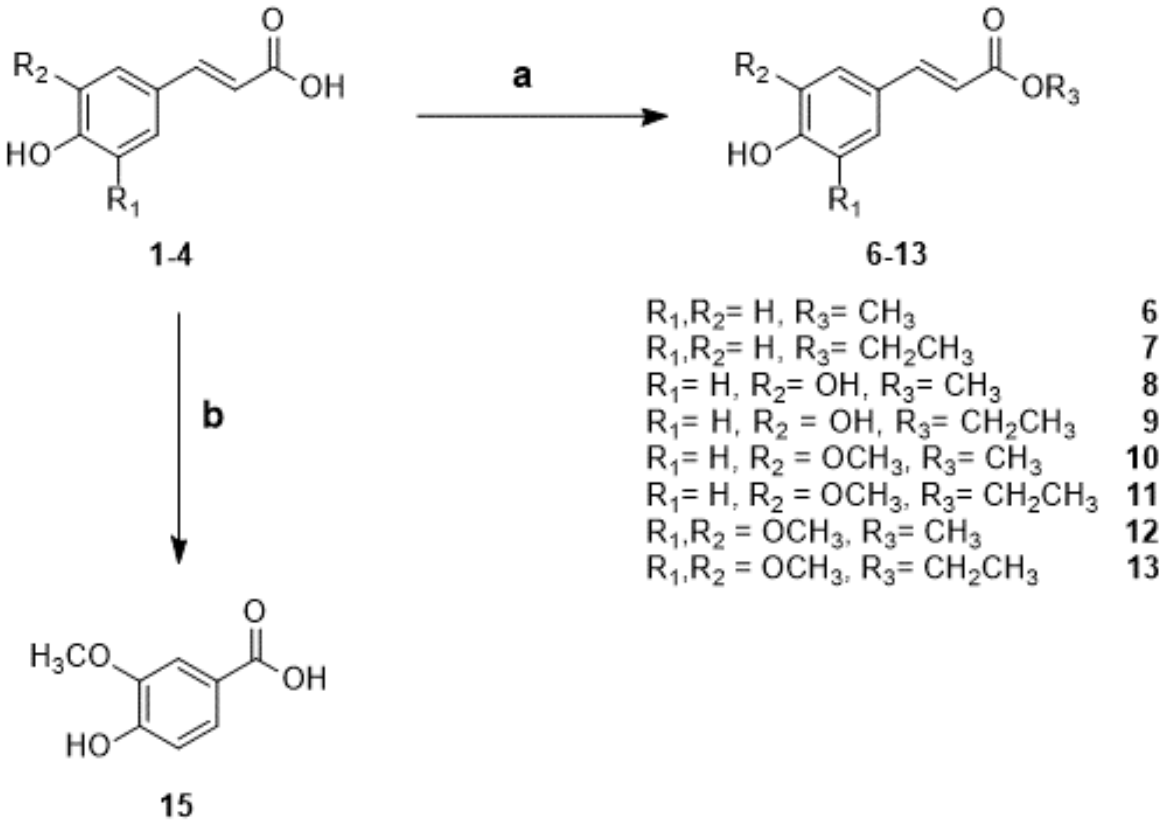


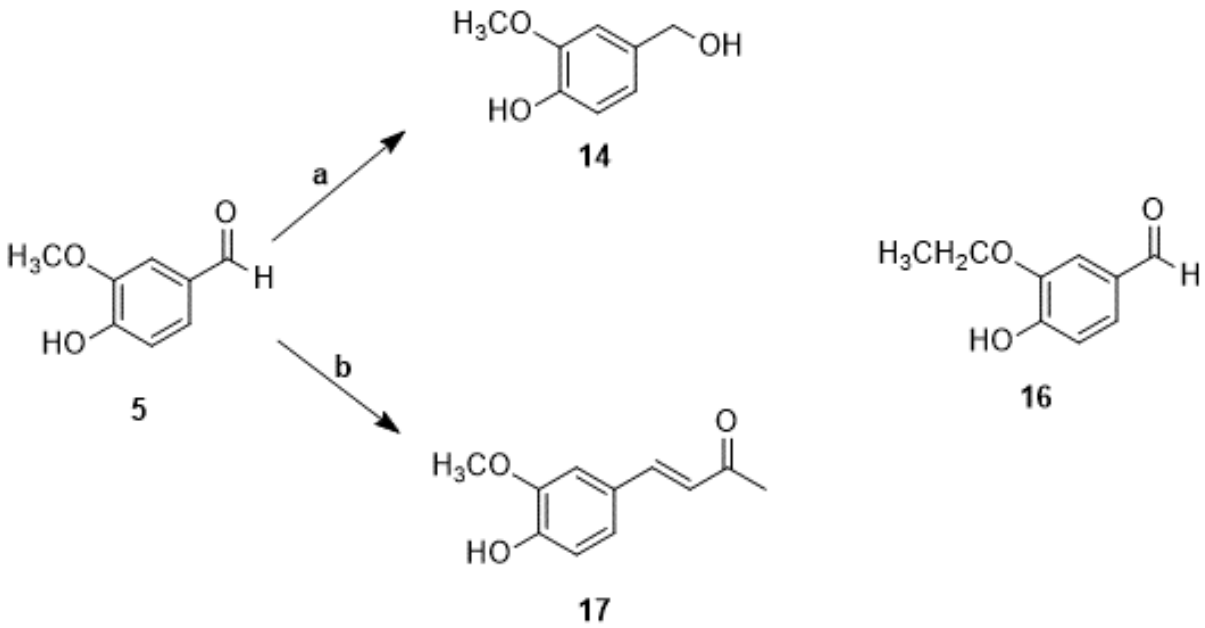
Figure 1. Hydroxycinnamic acids and vanillin structures.

Interestingly, searching for new peroxidase sources remains one of the leading research topics in the area in the last decade. Moussouni *et al*. (2011) used onion wastes showing peroxidase activity to dimerize methyl p-coumarate, caffeate and ferulate. Coconut water has also been proposed as a source of peroxidases to oxidize model phenolic compounds (Rodrigues *et al*., 2017). Sánchez-Carvajal *et al*. (2018) obtained dimers of ferulic acid using enzymatic extracts from *Opuntia ficus-indica* wastes. More recently, methyl esters of different hydroxycinnamic acids (6, 8, 10, 12, Scheme 1) have been tested as substrates in the synthesis of lignin-molecules using an enzymatic crude extract from onion (Palade *et al*., 2019).

The main objective of the present work is to show that *Raphanus sativus* var sativus was used as an accessible source of peroxidases with catalytic activity towards oxidative coupling of phenolic compounds to yield lignans in the form of dimeric precipitates. Such products were easy to isolate and thus did not require further purification steps. Methyl coumarate (6), ethyl coumarate (7), methyl caffeate (8), ethyl caffeate (9), methyl ferulate (10), ethyl ferulate (11), methyl sinapate (12), and ethyl sinapate (13) (Scheme 1); together with vanillin derivatives: vanillin alcohol (14), vanillic acid (15), ethylvanillin (16) and compound 17 were oxidized with a crude enzymatic extract (Scheme 2). Five major products were isolated after oxidative coupling in the form of dimeric precipitates and were fully characterized by ^{1}H and ^{13}C nuclear magnetic resonance (NMR). Moreover, one dimer was isolated after several purification steps.



Scheme 1. Synthetic pathway to obtain hydroxycinnamate esters through Fischer's reaction conditions. a. HCl, methanol (MeOH), or ethanol (EtOH), reflux, 24 h; b. *Aspergillus niger*, incubated 24 h.



Scheme 2. Synthetic strategies for the preparation of the vanillin derivatives subjected to oxidative coupling with radish peroxidase. a. $NaBH_4$/NaOH, 20 min; b. Acetone/EtOH, NaOH, 1 h

2 Materials and methods

2.1 Materials

Reagents were purchased from Sigma-Aldrich and used without further purification. Compounds synthesized before oxidative coupling were obtained as described here.

NMR spectra were recorded on an Agilent 600 DD2 (600 MHz) spectrometer in acetone-d_6, $CDCl_3$, or DMSO-d_6. HPLC analysis was carried out using a Hypersil Gold column (250 x 4.6 mm) using a Waters HPLC (pump: Waters 1525; UV/vis detector: Waters 2478). Gradient elution was applied (0-30 min 100% A, 30-40 min 48% A, 52% B, 40-50 min 100% B) with the mobile phase consisting of A: 4 % (w/w) acetic acid (AcOH) and B: AcOH/acetonitrile (AcN)/methanol (MeOH) (1:5:94) at a flow rate of 1 mL/min. Spectrophotometric assays were carried out using a GBC-Cintra 101 spectrophotometer at 380 nm.

2.2 General procedure. Synthesis of methyl and ethyl esters (6-13)

The esters 6-13 were synthesized through Fischer esterification of compounds 1-4 with the corresponding alcohol. The hydroxycinnamic acids (1 g) were added to methanol or ethanol (50 mL) and stirred until complete solubilization. Next, 5 mL of concentrated H_2SO_4 was added dropwise, and the reaction mixture was refluxed for 24 h with magnetic stirring. Upon reaction completion, the mixture was cooled to room temperature and neutralized to a pH of 7 with 0.1 M NaOH. Following the extraction with ethyl acetate (EtOAc, 3 x 20 mL), the organic layers were dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. Compounds 6, 8-9 and 13 were recrystallized from an acetone/H_2O mixture. Compounds 7, 10-12 were purified using silica gel chromatographic column (Hexanes/EtOAc, 80:20). All compounds 6-13 were identified by ^{1}H NMR and ^{13}C NMR and the data compared to that previously reported (Moussouni *et al*., 2011; Sánchez-Carvajal *et al*., 2018); for compounds 10-13 the corresponding shifts for the ethyl moiety were also determined. After isolation, the afforded yields were in the range of 50-90% (Table 1).

Table 1 The obtained yields of alkyl hydroxycinnamic esters.

Compound	Name	Yield (%)
6	Methyl coumarate	94
7	Ethyl coumarate	71
8	Methyl caffeate	60
9	Ethyl caffeate	58
10	Methyl ferulate	79
11	Ethyl ferulate	94
12	Methyl sinapate	79
13	Ethyl sinapate	93

2.3 Synthesis of vanillyl alcohol (14)

Vanillin (5 g, 32.9 mmol) was solubilized in the minimum amount of ethanol and transferred to a 50 mL round bottom flask. The resulting solution was cooled (0 °C) in an ice bath prior to gradual addition of a $NaBH_4$ solution (10 mL, 6.5 M) in NaOH (1M). The mixture was stirred for 20 min. After reaction completion, concentrated HCl was added until an acidic pH was obtained, and as no bubbling was noticed it was assumed that H_2 was not formed. The formed precipitate was filtered, washed with small portions of ice water and dried under vacuum, affording 14 (4.5 g, 29.2 mmol) as a white solid. Spectroscopic data was consistent with literature (Lai *et al*., 1985; Zhou *et al*., 2008).

2.4 Biosynthesis of vanillic acid (15)

Ferulic acid (1 g, 5.1 mmol) was incubated with *Aspergillus niger* mycelium as described previously (Lira Parada, 2014). A fermentation system was implemented using eight reactors which were inoculated with the necessary volume to achieve 10^8 spores per flask at 30 °C. After a 24 h incubation, the resulting biomass was filtered off and extracted according to the procedure of Andreoni *et al*. (1995).

2.5 Synthesis of compound (17)

Vanillin (1 g, 6.6 mmol) was solubilized in ethanol (9 mL) and acetone (1.9 mL, 26.3 mmol) was added. The resulting mixture was stirred vigorously. After complete homogenization, 8 mL of 8% NaOH (w/v) were added. Reaction progress was followed by TLC for 2 h. Following that time, 4 mL of 8% NaOH (w/v) were added along with acetone (1 eq., 6.6 mmol), and the mixture was left for another 1 h until a brownish solid appeared. The precipitate was isolated by vacuum filtration and rinsed with MeOH. Methanol layers were concentrated until dryness, affording 17 (0.7 g, 3.6 mmol). ^{1}H NMR (600 MHz, $CDCl_3$-d): ? 2.35 (s, 3H), 3.85 (s, 3H), 6.51 (d, 1H, J= 16 Hz), 6.80 (d, 1H, J=7.7 Hz), 7.02 (d, 2H, J= 7.9 Hz), 7.45 (d, 1H, J= 16 Hz). Yield: 55%.

2.6 Preparation of crude red radish peroxidase (RRP) extract

Vegetable material was acquired from a local market (Mexico City, Mexico). The radish was washed and peeled before being used. Taproots (60 g) were crushed and mixed with acetate buffer (pH 4) at 1:1 ratio. The resulting mixture was filtered through gauze at 4 ºC. Polyvinylpyrrolidone (3 g) was added to the mixture and stirred for 5 min. The extract was filtered through Celite bed (2 cm thick, previously washed with distilled acidic water (pH 4) and stored on ice before use. Protein parameters of RP extract were measured. Enzyme activity and specific activity were determined spectrophotometrically using catechol as the peroxidase substrate, as it oxidizes to quinone. Briefly, 20 μL of the extract were mixed with 3 mL of phosphate buffer (0.1 M, pH 5) and 1 mL of 1% catechol solution (v/v). Absorbance of the quinone existing in the solution was measured every 10 s for 3 min at 293 nm.

Total protein content was determined by the Bradford assay using bovine serum albumin as the standard and a Bradford reagent kit (Bio-Rad, USA). Briefly, 20 μL of the extract were mixed with 780 μL of phosphate buffer (0.1 M, pH 6) and 200 μL of Bradford reagent. Absorbance was measured at 595 nm. The results of total protein content, enzymatic activity, and specific activity are shown in Table 2. Enzymatic activity units (U) were defined as the amount of substrate oxidized per minute in standard conditions.

Table 2. Protein parameters of RP extract. U = oxidized substrate in μmol/min.

Total protein (g)	**Enzymatic activity** (U)	**Specific activity** (U/g)
9.4	839.1	89.4

2.7 Oxidative coupling (OC) of alkyl hydroxycinnamates and vanilloids. General procedure

To a stirring solution of radish extract (60 mL) in 100 mL Erlenmeyer flask the corresponding alkyl hydroxycinnamate or vanilloid was transferred. When needed, acetone was added as co-solvent to help solubilize the substrate. After complete homogenization, a 3% H_2O_2 solution (v/v) was added dropwise. The resulting mixture was stirred until a precipitate appeared or left for 30 min to react. Finally, a concentrated HCl solution was added until a pH of 3 was reached. The precipitate was separated by vacuum filtration, washed with acetate buffer (pH 4) and dried.

Compound 18. According to general procedure of oxidative coupling, compound 18 (6.4 mg, 0.02 mmol, oil, isolated yield: 11%) was obtained from 6 (302 mg, 1.7 mmol) after extraction with EtOAc (3 x 20 mL) and purification of the residue (53.7 mg) by preparative TLC. ^{1}H NMR (600 MHz, DMSO, d_6) δ 3.79 (s, 3H), 3.82 (s, 3H), 4.26 (d, 1H, J= 7.5 Hz), 6.08 (d, J= 7.5 Hz), 6.31 (d, 1H, J= 16 Hz), 6.82 (d, 2H, J= 8.7 Hz), 6.87 (d, 1H, J= 8.4 Hz), 7.25 (m, 2H), 7.41 (dd, 1H, J= 8.3 Hz), 7.56 (m, 1H), 7.65 (d, 1H, J= 15.9 Hz). ^{13}C NMR (150 MHz, DMSO, d_6) δ 54.3, 55.5, 57.7, 89.0, 113.0, 117.9, 118.3, 127.6, 130.1, 130.4, 132.5, 133.4, 147.3, 158.6, 163.8, 170.5, 173.5.

Compound 19. According to general procedure of OC, compound 19 (78.2 mg, 0.176 mmol, brown solid, isolated yield: 13%) was obtained from 12 (308 mg, 1.4 mmol). ^{1}H NMR (600 MHz, DMSO, d_6) δ 1.25 (m, 6H), 3.76 (s, 3H), 3.84 (s, 3H), 4.18 (m, 4H), 4.51 (d, 1H, J= 8.0 Hz), 5.91 (d, 1H, J= 8 Hz), 6.55 (d, 1H, J= 15.9 Hz), 6.77 (d, 1H, J= 8.0 Hz), 6.81 (dd, J= 8.1 Hz), 7.00 (s, 1H), 7.26 (s, 1H), 7.39 (s, 1H), 7.62 (d, 1H, J= 15.7 Hz). ^{13}C NMR (150 MHz, DMSO, d_6) δ 14.0, 14.2, 54.2, 55.6, 55.9, 59.8, 61.3, 87.2, 110.8, 112.4, 115.3, 115.7, 118.2, 119.3, 126.2, 128.1, 129.8, 144.3, 144.5, 147.1, 147.7, 149.4, 166.4, 170.2.

Compound 20. According to general procedure of OC, compound 20 (53.5 mg, 0.11 mmol, brown solid, isolated yield: 11%) was obtained from 13 (245 mg, 0.97 mmol). ^{1}H NMR (600 MHz, DMSO, d_6) δ 1.09 (t, 3H, J=7.1 Hz), 1.21 (t, 3H, J= 7.1 Hz), 3.60 (s, 3H),

3.75 (m, 6H), 3.83 (s, 3H), 4.01 (m, 4H), 4.13 (m, 1H), 4.81 (s, 1H), 6.20 (s, 2H), 7.05 (s, 1H), 7.63 (s, 1H). ^{13}C NMR (150 MHz, DMSO, d_6) δ 13.9, 14.1, 31.3, 46.1, 56.0, 56.0, 59.7, 60.1, 60.6, 105.0, 108.7, 121.8, 122.1, 123.1, 132.6, 134.5, 137.3, 141.8, 145.4, 147.7, 166.0, 171.3.

Compound 21. According to general procedure of OC, compound 21 (53 mg, 0.16 mmol, brown solid, isolated yield: 5%) was obtained from 16 (570 mg, 3.4 mmol). ^{1}H NMR (600 MHz, DMSO, d_6) δ 1.11 (s, 6H), 4.31 (m, 4H), 6.70 (br s, 2H), 6.72 (br s, 2H), 8.72 (s, 2H). ^{13}C NMR (150 MHz, DMSO, d_6) δ 14.5, 64.3, 110.3, 124.6, 127.8, 127.9, 147.2, 150.5, 191.2.

Compound 22. According to general procedure of OC, compound 22 (160 mg, 0.47 mmol, brown-red solid, isolated yield: 15%) was obtained from 15 (500 mg, 3.0 mmol). ^{1}H NMR (600 MHz, DMSO, d_6) δ 3.34 (s, 6H), 7.17 (m, 4H), 12.85 (s, 2H). ^{13}C NMR (150 MHz, DMSO, d_6) δ 55.7, 113.5, 122.8, 122.8, 123.4, 148.1, 150.1, 166.7.

Compound 23. According to general procedure of OC, compound 23 (247.3 mg, 0.81 mmol, brown-red solid, isolated yield: 25.6 %) was obtained from 14 (0.50 g, 3.2 mmol) ^{1}H NMR (600 MHz, DMSO, d_6) δ 3.79 (m, 6H), 7.35 (m, 4H), 9.82 (m, 2H). ^{13}C NMR (150 MHz, DMSO, d_6) δ 56.0, 109.1, 124.6, 127.7, 128.1, 148.1, 150.4, 191.3.

3 Results and discussions

Although dimerization of the phenolic compounds with HRP has been already demonstrated and widely applied, to the best of our knowledge, no previous work has been published about the use of RRP as a biocatalyst in the oxidative coupling reaction. The corresponding hydroxycinnamic acid derivatives, i.e. alkyl hydroxycinnamates (6-13), and vanillin derivatives, i.e. vanilloids (14-17), were tested as model compounds in the oxidative coupling reaction to obtain lignan-type dimers that easily precipitate in a single isolation step. It should be noted that even though these are free radical-mediated reactions, the data obtained and presented in the manuscript did not show or suggest the formation of polymeric structures.

Treating 6-9 with the crude enzyme extract provided their respective dimers as the main products (Figure 2). Dimers of ethyl ferulate (19) and ethyl sinapate (20) were easily recovered as both precipitated to provide 12% and 11% yield, respectively. Meanwhile, the main product of dimerization of methyl coumarate (18) was isolated by preparative thin layer chromatography in 11% yield after a 30 min reaction with no precipitate observed. Compounds were identified by ^{1}H and ^{13}C NMR and compared with the literature. The corresponding dimers of 18 and 19 were compared with dehydrobenzofuran skeleton and the E enantiomer described by Moussouni *et al*. (2011), and dimerization product of 20 was compared with dehydronaphtalene skeleton reported by Setälä *et al*. (1994). It resulted very interesting that 18 and 19 presented the same stereochemistry, as showed when spectroscopic data was compared with previous reports (Moussouni *et al*., 2011).

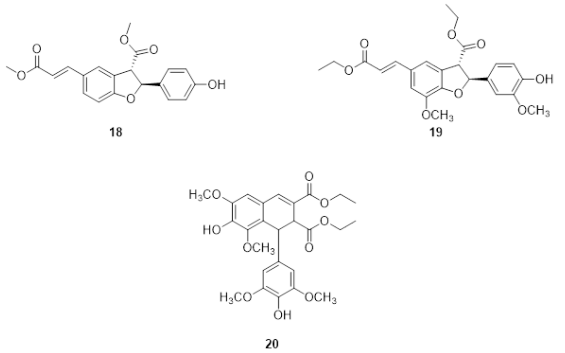


Figure 2. Chemical structures of the dimers isolated after enzymatic oxidation of alkyl hydroxycinnamates with crude RRP.

The reaction of methyl coumarate (6) and methyl ferulate (8) resulted in the production of dehydrobenzofuran. Moussoni *et al*. (2011) proposed a reaction mechanism in which two different radicals are initially formed: a C-8 centered semiquinone and a C-5 centered quinone methide. The latter produces a new C-C bond which undergoes regioselective cyclization. The final product of this nucleophilic attack is the resulting dehydrobenzofuran structure. Likewise, the formation of dehydronaphtalene begins with the coupling of two C-8 semiquinone radicals that form a bisquinone methide intermediate. Then, intramolecular nucleophilic attack of C-2 to C-7' of the bisquinone methide triggers cyclization which affords dehydronaphtalene structure (Andreoni *et al*., 1995).

The optimum conditions for the oxidative coupling of hydroxycinnamates has long been known to strongly depend on the reaction pH and the use of co-solvents. In this study, the corresponding dimers of compounds 10 and 12 were obtained as precipitates

at pH 4. This pH has been described as optimal to control the reaction progress towards dimer formation and avoid oligomer production as well as other side-products of methyl ferulate and methyl sinapate (Andreoni *et al*., 1995; Moussouni *et al*., 2011). However, for the dimerization of methyl *p*-coumarate (6) and methyl caffeate (8), the optimum pH was found to be 5 and 6, respectively. Nevertheless, different oxidative coupling products were formed, and laborious isolation procedures had to be applied, such as in the case of dimer (18). We hypothesized that the aromatic substitution pattern plays a significant role in establishing the optimal pH conditions, mainly the presence of the *ortho*-methoxy groups.

As mentioned previously, co-solvents play a key role in the outcome of dimerization reactions. For example, MeOH and glycerol have been used to successfully synthesize dimers of methyl *p*-coumarate and methyl ferulate, respectively, while dimethyl formamide (DMF) was used for methyl caffeate oxidative coupling (Moussouni *et al*., 2011; Palade *et al*., 2019). In this study, aqueous acetone favored the formation of dimer 20 as was also reported by Setälä *et al*?. (1994). However, no benefit was observed for the dimerization of eugenol with the addition of acetone, dioxane or tetrahydrofuran (Sánchez-Carvajal *et al*., 2018)?. Nevertheless, our findings are consistent with the reports mentioned above for the application of polar and aprotic solvents for dimerization of ethyl sinapate and the use of polar and protic co-solvents to obtain dimers over oligomers of alkyl *p*-coumarate, caffeate and ferulate.

Vanilloids 14-16 were tested as substrates for dimerization catalyzed by the crude RRP enzyme. Three main dimer products were isolated after precipitation in the reaction media (21-23; Figure 3). For the remaining vanilloids tested, products were neither isolated as precipitates, nor could be identified following the separation on preparative thin layer chromatography (Table 3). Compounds 21 and 22 were isolated in 5 and 15% yield, respectively. The ^{13}C-NMR analysis was significantly easier than that of the phenylpropanoid derivatives due to the symmetry of the products. It revealed the formation of dimers 21-23 that were coupled in *ortho-ortho* position, as confirmed by the characteristic chemical shift at ~124 ppm (Antoniotti *et al*., 2004; Nishimura *et al*., 2010; Enomoto & Iwata, 2020).

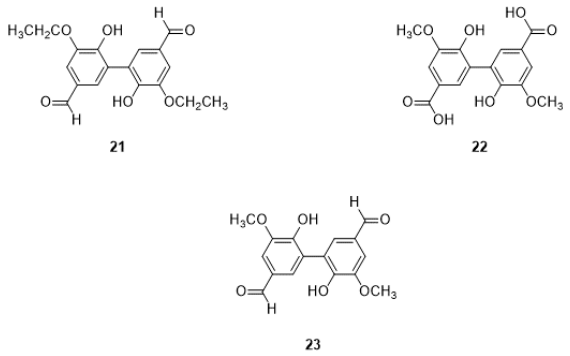


Figure 3. Chemical structures of dimers isolated after oxidizing of vanilloids with RPP preparation.

Furthermore, dimer 21 showed characteristic chemical shifts for the methylene of the ethoxy moiety as the multiplet at 4.31 ppm and two broad singlets for the four aromatic hydrogens at 6.70 and 6.72 ppm on ^{1}H NMR spectra respectively. Complementary to proton analysis, ^{13}C NMR spectra revealed characteristic signals for the methylene carbon at 64.34 ppm, and the aldehyde carbon was identified at 191 ppm. As for dimer 22, the prototypical chemical shifts for six hydrogens of the methoxy moiety were observed as a singlet at 3.34 ppm, and two hydrogens of the carboxylic acid residue were assigned as a singlet at 12.85 ppm on ^{1}H NMR spectra. The carboxylic acid carbon at 166.74 ppm was observed on ^{13}C NMR spectra.

Preparation of compounds such as 21 and 23 via this methodology could represent a very convenient route, as these dimers present remarkable pharmaceutical applications. Jantaree *et al*. (2017) tested both, 5,5'-divanillin and 5,5'-diapocyn, for the inhibition of focal adhesion kinase (FAK), an enzyme involved in a downstream process that triggers metastasis of liver cancer cells. Experimentally authors found that dimers were better inhibitors than their respective monomers. In that same report, authors used molecular modelling for better understanding of this inhibition, and compared interaction cites of these dimers with a known FAK inhibitor (I; 1,2,4,5-benzenetetraamine hydrochloride). Divanillin and diapocyn bind tightly to the very same domain than the inhibitor, thus preventing its phosphorylation. Their work showed that diapocyn binds to the domain through 5 hydrogen bonds, forming a very stable supramolecular structure that helps prevent metastasis.

Table 3. Isolated dimers after oxidative coupling of phenolic compounds with RP preparation. ND: Not Determined.

Substrate	Dimer	Name	Yield (%)
6	18	methyl (*E*)-2-(4-hydroxyphenyl)-5-(3-methoxy-3-oxoprop-1-enyl)-2,3-dyhidrobenzofuran-3-carboxilate	11
7	ND	—	—
8	ND	—	—
9	ND	—	—
10	ND	—	—
11	ND	—	—
12	19	ethyl (*E*)-2-(4-hydroxy-3-methoxiphenyl)-7-methoxy-5-(3-methoxy-3-oxoprop-1-enyl)-2,3-dyhydrobenzofuran-3-carboxilate	13
13	20	diethyl 7-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-6,8-dimethoxy-1,2-dyhydronaftalen-2,3-dicarboxilate	11
14	23	6,6'-dihydroxy-5,5'-dimethoxybiphenyl-3,3'-dicarbaldehyde	26
15	22	6,6'-dihydroxy-5,5'-dimethoxy-[1,1'-biphenyl]carboxylic diacid	15
16	21	6,6'-dihydroxy-5,5'-diethoxybiphenyl-3,3'-dicarbaldehyde	5
17	ND	—	—

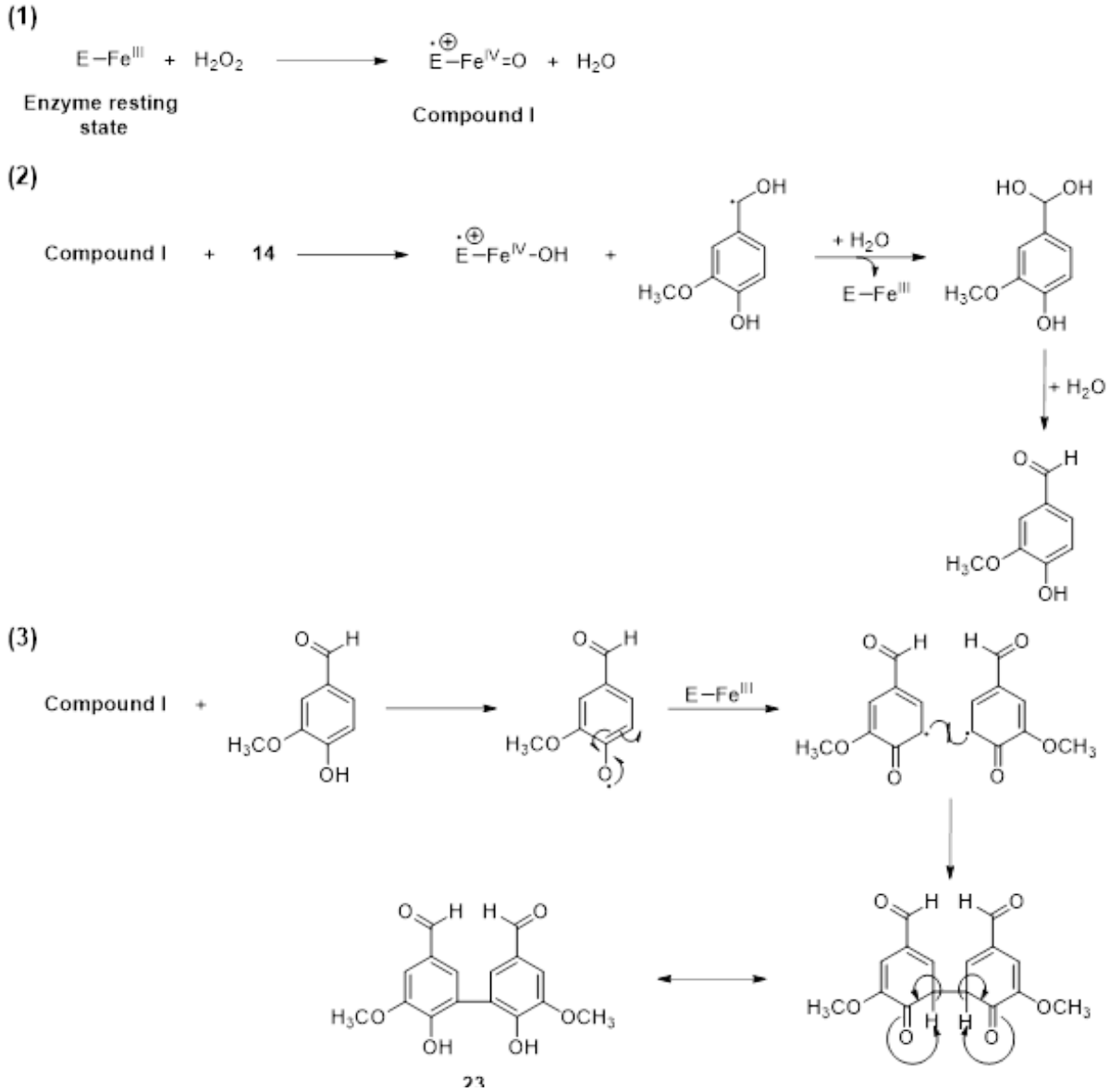
Although the peroxidase-catalyzed synthesis of dimers 21 and 22 have not been reported yet, vanillin and related derivatives (apocynin, 4-methylguaiacol) have been subjected to the dimerization reaction using soybean peroxidase at low pH (Antoniotti *et al.*; 2004). The expected products were afforded at 2-32% yields as main products. Amarasekara *et al.* (2012) reported the synthesis of divanillin with HRP at 95% yield which is in accordance with those obtained by Antoniotti *et al.* (2004) for vanillin, apocynin and 4-methylguaiacol. It was suggested that electronic effects determine the coupling type in the way that electron-donating substituents favor primarily ortho-ortho coupling, as well as dimer formation over higher oligomers. Furthermore, to explain low yields obtained for some substrates, it was suggested that electron-withdrawing *para*-substituents, like the aldehyde and carboxylic acid moieties, could deactivate the reaction.

Another factor that could be consider is the possible inactivation of the enzyme. There is experimental evidence that suggests that this type of peroxidases could be inactivated because of a covalent bond formation between the substrate and the enzyme (Huang *et al*., 2005; Kim *et al*., 2009). First, Huang *et al.* (2005) reported that phenoxy radicals inhibited the activity of commercial HRP. Then, Kim *et al.* (2009) observed that some phenolic derivatives, among them vanilloids and phenylpropanoids, when forming radical moieties, tend to inactivate a fungal peroxidase. This effect was found to be a consequence of the creation of a supramolecular structure through the formation of a covalent bond between phenoxy radicals and aromatic aminoacids, such as Phe, following an *ortho-ortho* coupling mechanism. Although high inactivation activity was found with phenol, *p*-hydroxybenzaldehyde and different cresols, phenylpropanoids and vanilloids only presented moderate to negligible inhibitory activity.

Noteworthy, an unexpected product was observed during the oxidation of compound 14, and instead of the corresponding dimer, compound 23 was obtained (26% yield; Table 3). Zhi *et al.* (2008) previously suggested the oxidation of primary alcohols when using HRP. Thus, we propose a similar mechanism for the oxidation of vanillyl alcohol when testing its dimerization (Scheme 3). First, Fe (III) of the protoporphyrin heame-group in the active site of peroxidase is oxidized with H_2O_2 to form an iron (IV)-oxo complex of the porphyrin (E) radical cation (compound I in Equation 1, Scheme 3). In the next step (Equation 2, Scheme 3), this radical species abstracts the proton from the C_α of vanillyl alcohol (14) which leads to the formation of an iron (IV)-hydroxy complex of the porphyrin (E) radical cation and a benzylic radical. The latter quickly undergoes a rearrangement to aldehyde 5 (through the hemiacetal intermediate) in the presence of water and is therefore catalyzed by the enzyme which returns to its resting state (Fe III). Additionally, we suggest peroxidase maintains enzymatic activity to catalyze oxidative coupling of the newly formed vanillin (5, Equation 3, Scheme 3). Briefly, compound 5 reacts with the

activated form of peroxidase producing an *ortho* radical species which couples to form the *ortho-ortho* dimer (compound 23).

When phenylpropanoids and vanilloid dimers are compared, it is remarkable that the latter present a clear symmetry, while the former do not. Rodrigues *et al*. (2017) suggested that when HRP is used in its commercial preparation, the reaction loses stereoselectivity, thus forming a mixture of isomers that includes asymmetric ones. Authors claimed that some synthases are naturally present along with peroxidases, but when the latter is purified for commercial purposes, loses such auxiliary enzymes. These synthases are thought to play an important role in the alignment of phenoxy radicals generated by the action of peroxidases. In our case, it could be possible that these protein-aides are necessary only for the alignment of phenylpropanoids and not the vanilloid derivatives, and presumably these additional enzymes were not present in the crude preparation.



Scheme 3. Proposed mechanism for the vanillin alcohol conversion to vanillin and oxidative coupling to form dimer 23.

The current study demonstrated that *Raphanus sativus var. sativus* extract presents peroxidase activity towards the oxidative coupling of phenolic compounds. The reaction strongly depends on the reaction medium, as well as the chemical structure and electronic effects of the substrates used. The important disadvantage of the application of crude peroxidase in this study is that it could contribute to low reaction yields, lack of separable dimers of some phenolic compounds tested here, and unexpected products. It was also curious that, depending on the starting compound structures, the outcome of dimerization reactions was different. When hydroxycinnamate derivatives were used, predominantly asymmetric dimers were prepared, although with a specific stereochemistry. While the use of vanilloids garnered entirely symmetric derivatives. These might as well could be due to the length of the propanoid chain of cinnamates. Although there is enough evidence of the formation of symmetric dimers of phenylpropanoids, such as 5-5' of ferulic acid and others. It would be very interesting to take advantage of the computational tools at hand to help determine the nature of the interaction between the substrate and the enzyme, and thus explain product structures. This has been recently reported with related enzymes such as laccase and veratryl alcohol oxidase (Herrera-Zúñiga *et al*., 2021).

Finally, protein parameters measured for the extract used in this work had lower specific activity (0.09 U/mg) in comparison to 12.51 U/mg of crude enzymatic extract of *Opuntia ficus-indica* cladodes (Sánchez-Carvajal *et al*., 2018), even though higher than that already reported for radish (Zhi *et al*., 2008). Similarly, the specific activity of brown-skin onion bulb homogenate assessed at 0.11 U/mg was improved in a concentrated (Rathnamsamy *et al*., 2014) and partially purified extract (Moussouni *et al*., 2011). Accordingly, Palade *et al*. (2019) reported the dimerization of methyl ferulate using a crude peroxidase from onion bulbs. Finally, Osman *et al*. (2008) and Sánchez-Carvajal *et al*. (2018) highlighted the fact that the raw extract of peroxidases consists of several peroxidase isoforms and can contain other enzymes capable of altering the expected peroxidase activity during its utilization. Nevertheless, a possible inactivation due to the linkage between phenoxy radicals and the enzyme could play a role in the low yields observed (Huang *et al*., 2005; Kim *et al*., 2009).

Conclusions

It has been demonstrated that red radish *Raphanus sativus var sativus* presents peroxidase activity as it can accomplish regioselective oxidative coupling of the phenolic compounds to dimers. Notable, vanilloid dimers formed through an *ortho-ortho* coupling gave symmetrical molecules. However, due to the crude RRP preparation used in the experiments, low yields were obtained: 12% and 26% for alkyl hydroxycinnamates and vanilloids substrates, respectively. Future work will expand the scope of phenolic substrates, focus on the effects of co-solvents on the reaction outcome, and apply purified peroxidase for better understanding of the enzyme-catalyzed oxidative coupling reaction and to improve reaction yields. Although testing the possible inactivation of the enzyme due to the formation of a covalent bond between radicals and aromatic aminoacids residues could give more insight into the outcome of these reactions.

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