Short Note

2,3-Bis((*E*)-4-hydroxybenzylidene)-*N*1,*N*4-bis(4-methylbenzyl)succinamide

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Abstract: Lignans and neolignans are dimeric natural products with an extraordinary variety of structures and biological properties. Diphenylbutadienes are a subclass of lignans rarely found in nature with cannabisin G being the most representative example. This lignan, found in *Cannabis sativa* seed, has shown anti-inflammatory and antioxidant activity among other biological properties. Different methodologies have been reported for the synthesis of cannabis G to be employed in new biological studies. We report herein a green and concise procedure based on the use of *Trametes versicolor* laccase for the synthesis of a new diphenylbutadiene. The developed procedure may be employed for the synthesis of cannabisin G and other analogues.

Keywords: lignans; biocatalyst; laccase; synthesis

1. Introduction

Lignans and neolignans are a group of dimeric natural products biosynthesized through oxidative coupling involving phenylpropanoid units C6C3. In the plant kingdom these products are secondary metabolites with an extraordinary variety of structures and biological properties [1]. Specifically, oxidases, namely laccases, peroxidases and cytochromes, are involved in the formation of phenoxy radicals C6C3 yielding dimeric products with different structures [2]. According to IUPAC, lignans are dimeric products obtained by coupling at position 8-8′ of two phenoxy radicals; differently, oxidative coupling in other positions gives rise to neolignans dimers. The biological properties of lignans and neolignans are well documented as they have shown antioxidant, anti-inflammatory, cardiovascular, and antitumor activities. Diphenylbutadienes are a subcategory related with the arylnaphatalene type lignans and are rarely identified in plants. Cannabisin G is the most representative example, firstly isolated from the fruits of *Cannabis sativa* (Figure 1) [3]. It's accepted that it is biosynthesized from oxidative coupling of *N-trans*-feruloyltyramine and is also classified as a lignanamide for the presence of amide functions [4]. It is reported to have anti-inflammatory activity as an inhibitor of NO production [5], in vitro DPPH radical scavenging and a protective effect from hydroxyl radical scavenging activity [6]. Cannabisin G has also shown cytotoxic activity against human prostate cancer LNCaP cells [7].

In 2014, two other lignanamides (**1** and **2**), demethylated cannabis G products, were isolated from *Cannabis sativa* seeds (Figure 1) [8]. In 2020, cannabisin G was also isolated from *Lycium barbarum* seeds, a medicinal food largely consumed in Asian regions [9]. Cannabisin G together with known and newly identified lignans/neolignans were evaluated for their immunological activity in an immunodeficiency mouse model [9].

Synthetic efforts to achieve cannabisin G have been developed according to the biosynthetic pathway based on oxidative coupling of *N-trans*-feruloyltyramine. However, these biomimetic strategies lack selectivity and often give rise to scant yield desired products and a large number of other dimeric compounds as by-products.
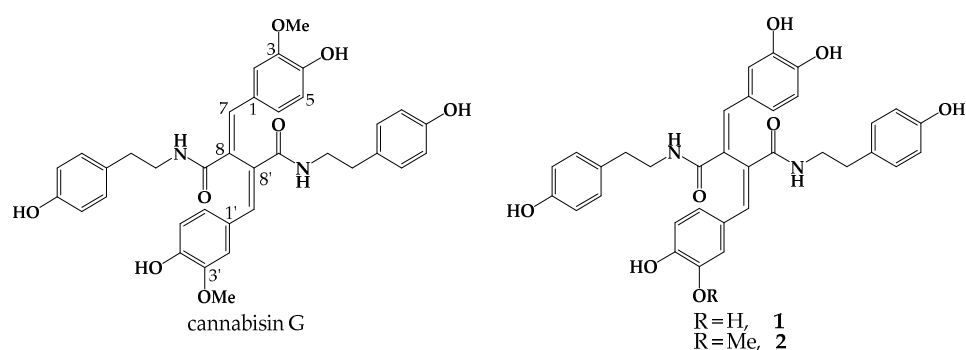
Figure 1. Example of naturally occurring diphenylbutadiene lignans. Cannabisin G and demethylated derivatives **1** and **2**.

In 2010 Xia et al., reported the synthesis of cannabisin G starting from vanillin and employing Stobbe condensation followed by the condensation of the obtained diacid derivative with tyramine [10]. The lignanamide was achieved with an overall 22.3%. In a different synthetic approach, Li et al., proposed a concise and highly regioselective synthesis with potassium ferricyanide and cannabis G was obtained with 45% yield over four steps [4]. As a continuation of our efforts in the synthesis of dimeric compounds inspired by natural ones [11,12], we report herein the biomimetic synthesis of a new diphenylbutadiene lignanamide obtained with an environmentally friendly reaction based on the use of *Trametes versicolor* laccase.

2. Results and Discussion

Laccases (EC 1.10.3.2) are a group of oxidative enzymes widespread in plants, bacteria and fungi. They have been reported as enzymes able to catalyze the oxidation of low molecular weight organic compounds, especially phenols [1,2]. The repertoire of obtainable structures in nature is extensive. On the laboratory scale, these enzymes can be employed in radical coupling reactions of phenols to yield dimerization products, namely lignans and neolignans, and/or oligomers [13,14]. It is known that in the laboratory, the formation of dimeric phenolics in the presence of laccases or of other oxidative agents occurs with the same mechanism (phenoxy radical formation and coupling) through which plants biosynthesize lignans and neolignans [13]; thus, these reactions mimic the natural ones. During our investigations in the reactivity of oxidases to gain new bioinspired phenolic compounds, we have found that among all laccases, *Trametes versicolor* (TvL) is the best enzyme able to catalyze the formation of neolignans (8-5′ dimers) with a dihydrobenzofuran skeleton starting from cinnamoyl amides as substrates [12]. Furthermore, we have also observed that when *p*-coumaroyl amide **3** was treated with TvL in the conditions reported in Scheme 1, another product is formed, in addition to the expected neolignan **4**. After purification, the new product was analyzed by means of HRMS spectrometry and by NMR. MS analysis gave a *m/z* of 533,2488 imputable to [M + H]$^+$ pseudomolecular ion. The mass value twice that of amide **3** less 2 uma was in agreement with the formation of a dimeric product.

The analysis of ^{1}H and ^{13}C NMR spectra suggested the possible formation of a symmetric dimer consistent with the low number of observed signals. In particular, the protons signals were similar for number and chemical shift to those of amide **3**, except for the absence of the two doublets typical of *trans*-olefinic protons. Similarly, in the carbon spectrum, were easily detected the signal for amide carbon at δ 167.1, the signal for sp^3 methylene in α-position to NH amide linkage at δ 44.3. This last was correlated in the HSQC spectrum with the two double doublets at δ 4.85 and 4.50 imputable to diastereotopic protons. The signals for CH of aromatic rings (δ 133.2, 129.9, 128.0 and 117.2) were assigned thanks to the HSQC and HMBC correlations (Figure 2). The most interesting signal in carbon and proton spectra were that at δ 141.5 and the singlet at δ 8.42 which correlated in the HSQC spectrum. Moreover, the carbon signal (δ 141.5) correlated in the HMBC spectrum with the doublet at

δ 7.81 assigned to aromatic protons of coumaroyl moieties (H-2/2′ and H-6/6′). In turn, the singlet at δ 8.42 showed a HMBC correlation with amide carbon signal at 167.1. Based on this analysis, the resonances at 141.5 in the carbon spectrum and the proton signal at 8.42 were assigned to sp^2 CH in β-position to amidic functions (C-7/7′). Other key HMBC correlations (Figure 2) supported the formation of a dimer with the structure **5** reported in the Scheme 1. Although compound **5** is a secondary product, it is obtained with 32% yield.
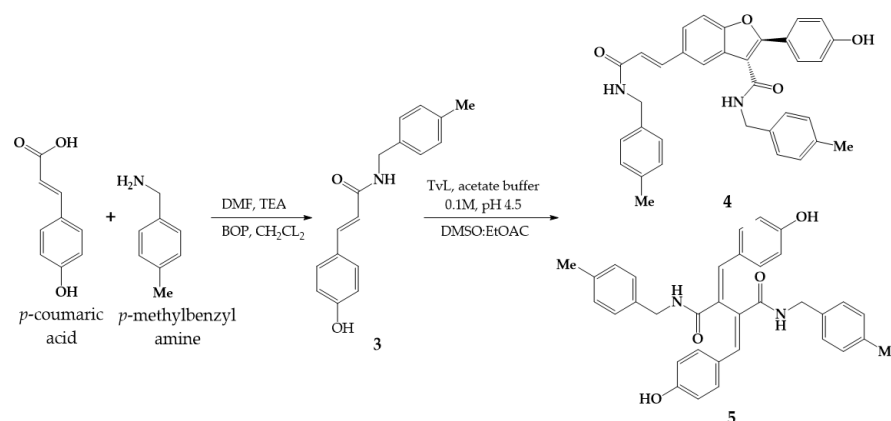
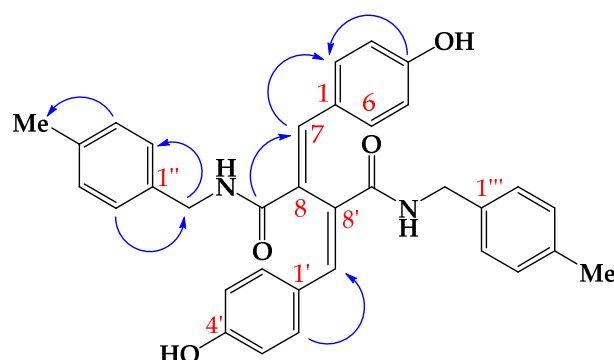**Scheme 1.** Synthesis of diphenylbutadiene lignan **5** and dihydrobenzofuran **4**.

Figure 2. Key HMBC correlations (from carbon to proton signals).

Worth of note, the dimer **5** have the same structure of cannabisin G, a bioactive dimer found in *Cannabis sativa*, which is known to be biosynthesized by oxidative coupling of *N-trans*-feruolyltyramide. This data suggested that the formation of **5** starting from amide **3** occurred with the same mechanism employed by plants. Moreover, the present synthesis can be employed to achieve with green conditions cannabisin G and other analogues simply employing the proper amide in the presence of TvL, with overall yields around 27%, comparable to that reported in the literature in other procedures [4,10]. Moreover, compared to those syntheses, this protocol employs less reaction steps and is concise as based on the use of two very simple reactions: (1) amidation and (2) green oxidative coupling mediated by TvL in a biphasic system.

3. Materials and Methods

All chemicals were of reagent grade and were used without further purification. *p*-Coumaric acid, *p*-methylbenzylamine, 1*H*-benzotriazol-1-yloxy)[tris-(dimethylamino)]phosphonium hexafluorophosphate (BOP) and the enzyme *Trametes versicolor* laccase (TvL, 10 U/mg) were purchased from Sigma Aldrich (Milan, Italy). Preparative liquid chromatography was performed on silica gel (63–200 μm, Merck, Darmstadt, Germany), using dichloromethane and methanol as eluent system. NMR spectra were run on a Varian Unity Inova spectrometer (Milan, Italy) operating at 499.86 (¹H) and 125.70 MHz (¹³C), and equipped with a gradient-enhanced, reverse-detection

probe. All NMR experiments, including g-COSY, g-HSQCAD, and g-HMBCAD, were performed at a constant temperature (300 K) in pyridine-d_5.

High-resolution mass spectra were acquired with a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with an ESI ion source operating in positive ion mode. Compound **5** was dissolved at 1×10^{-5} M concentration in 50:50 (MeOH/H$_2$O + 1% formic acid) and directly infused in the mass spectrometer. A survey scan was performed from *m/z* 150 to 1000 at 140 k resolution.

Synthesis of Lignanamide 5

Synthesis of N-trans-coumaroyl-4-methylbenzylamine (3). Amide **3** was synthesized as previously reported. Briefly, *p*-coumaric acid (200 mg, 1.21 mmol) was dissolved in dry dimethylformamide (DMF, 10.5 mL) and freshly distilled triethylamine (1 equiv) was added to the solution. The mixture was stirred at 0 °C for 10 min, then *p*-methylbenzylamine (215.8 mg, 1.6 mmol) was added. A solution of BOP in CH$_2$Cl$_2$ (534.8 mg, 1 equiv, in 20 mL) was dropwise added with a syringe within 30 min, the mixture was stirred at 0 °C for 30 min and at room temperature overnight. The crude of reaction mixture was concentrated in vacuo to remove CH$_2$Cl$_2$. The residue diluted in EtOAc (20 mL) was partitioned with 1 N HCl (2 × 10 mL), then with saturated NaHCO$_3$ (2 × 10 mL), and finally, the organic layer was washed with water. The organic residue taken to dryness was purified by flash chromatography on silica gel eluted with dichloromethane: methanol (99:1 → 90:10) to yield the expected amide **3** with 85% yield. Spectroscopic data were in agreement with those previously reported [12].

Synthesis of 2,3-bis-(4-hydroxybenzylidene)-N^1,N^4-bis(4-methylbenzyl)succinamide (5). Amide **3** (150 mg, 0.56 mmol) was dissolved in DMSO (1 mL) and diluted with EtOAc (67 mL). The organic solution was mixed with a TvL solution prepared in 0.1M acetate buffer (130.8 mg of TvL in 65 mL; pH 4.5) at rt for 24 h. The organic layer was separated from the aqueous layer, and the latter was partitioned with EtOAc (2 × 30 mL). Then, the total organic phase was washed with water and dried. The crude mixture was purified by flash column chromatography on silica gel with dichloromethane: methanol (100:0 → 93:7), and the lignanamide **5** was obtained with 32% as a white amorphous powder. R$_f$ 0.24 (CH$_2$Cl$_2$: MeOH 94:6). ^{1}H NMR (500 MHz, pyridine-d_5): δ 8.59 (bt, 2H, NH), 8.42 (s, 2H, H-7/7′), 7.81 (d, *J* = 8.2, 4H, H-2/2′ and H-6/6′), 7.08 (bs, 12H, H aromatic), 4.85 (dd, *J* = 15.2, 6.6 Hz, 2H, CONH-C*H$_a$*-H$_b$), 4.50 (dd, *J* = 15.2, 5.3 Hz, 2H, CONH-CH$_a$-*H$_b$*), 2.25 (s, 6H, CH$_3$); ^{13}C NMR (125 MHz, pyridine-d_5): δ 167.2 (C, CONH), 161.0 (C, C-4/4′), 141.5 (CH, C-7/7′), 137.6 (C, C-1″/1‴, C-4″/4‴), 133.2 (CH, C-2/2′, C-6/6′), 129.9 (CH, C-3″/3‴, C-5″/5‴), 128.0 (C, C-1/1′; CH, C-2″/2‴, C-6″/6‴), 127.6 (C, C-8/8′) 117.2 (CH, C-3/3′, C-5/5′), 44.3 (CH$_2$, CONH-CH$_2$-), 21.6 (CH$_3$, aryl-CH$_3$). The NMR data are listed in Table S1.

4. Conclusions

A new diphenylbutadiene lignan, structurally related to cannabisin G was obtained with an environmentally friendly procedure based on a condensation between *p*-coumaric acid and *p*-methylbenzylamine, followed by an oxidative coupling of the amide mediated by *Trametes versicolor* laccase, thus yielding the 8-8′ dimer with diphenylbutadiene skeleton with overall 27% yield.

Supplementary Materials: The following supporting information can be downloaded online. Figures S1–S7. MS and NMR spectra of compound **5**; Table S1 NMR data of compound **5**.

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Sample Availability: Samples of the compounds are not available from the authors. If requested, samples can be synthesized by the authors.

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