

Arylnaphthalene Lignans with Anti-SARS-CoV-2 and Antiproliferative Activities from the Underground Organs of *Linum austriacum* and *Linum perenne*

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Cite This: <https://doi.org/10.1021/acs.jnatprod.2c00580>

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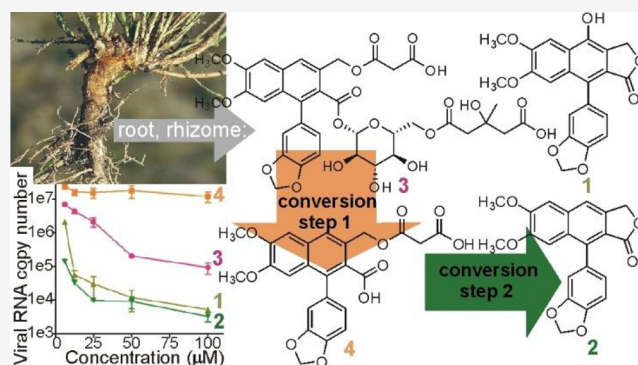
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ABSTRACT: Diphyllin (**1**) and justicidin B (**2**) are aryl-naphthalene lignans with antiviral and antiproliferative effects. Compound **1** is also known as an effective inhibitor of the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). To evaluate the *in vitro* antiviral and cytotoxic potency of both lignans in SARS-CoV-2-infected cells and various cancer cell lines, respectively, **1** and **2** were isolated from the underground organs of *Linum austriacum* and *Linum perenne*. Two previously undescribed aryl-naphthalene lignans, denominated linadiacin A and B (**3** and **4**), were also isolated and identified. In acidic media, **3** was converted by a two-step reaction into **2** via the intermediate **4**. Optimum acid treatment conditions were determined to isolate lignans by one-step preparative high-performance liquid chromatography (HPLC). The results of the conversion, HPLC-tandem mass spectrometry, nuclear magnetic resonance spectroscopy, and molecular modeling studies allowed complete structure analysis. Compounds **1** and **2** were the most effective against SARS-CoV-2 with a 3-log reduction in the viral copy number at a 12.5 μM concentration. Ten human cancer cell lines showed sensitivity to at least one of the isolated lignans.



Lignans constitute a large group of plant secondary metabolites biosynthesized by the dimerization of phenylpropanoid units. These compounds show high structural diversity as a result of different degrees of oxidation in the side chains, various substitutions on the aromatic rings, and unsaturation of the C7–C8 and C7'–C8' bonds.¹ Diphyllin (**1**) and justicidin B (**2**) are aryl-naphthalene lignans characterized by unsaturated C7–C8 and C7'–C8' bonds.

These two closely related lignans differ only in that **1** is hydroxylated at the C7 position while **2** does not contain any hydroxyl group.^{2,3} The free hydroxyl of **1** allows biotransformation with saccharides forming a glycosidic linkage; thus, diphyllin can also be accumulated in its glycosidic form.²

Compounds **1** and **2** have a wide range of pharmacological effects, including antiviral and antiproliferative activities. They are effective *in vitro* antiviral agents at submicromolar concentrations against various viruses, with low toxicity in the host cells used to propagate the viruses (Supporting Table S1).^{4–9} Compound **1** also presents antiviral activity against Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), without expressing any toxicity against the host cells Vero and Vero E6 (both are kidney epithelial cells derived from *Cercopithecus aethiops*).⁸

The antiproliferative activity of **1** in the esophageal cancer cell lines ECA-109 and TE-1 has been confirmed, with 50% inhibitory concentrations (IC_{50}) of 0.28 and 0.21 μM .¹⁰ Similarly, **2** demonstrates antiproliferative activity in the human gastric cancer line BGC-823 and the multiple myeloma cell line RPMI-8226, with IC_{50} values of 0.18 and 0.17 μM , respectively.^{11,12} The anti-SARS-CoV-2 and antiproliferative effects of **1** have been explained by its vacuolar ATPase (V-ATPase) inhibitory activity.^{8,10}

The treatment of a significant proportion of tumors and viral infections has remained unresolved. Moreover, the emergence of resistance to already used medicaments is a challenging problem. Thus, identifying new drug candidates against viruses and tumors is of primary importance. The results detailed above justify further studies to evaluate the antiviral and antiproliferative potential of **1**, **2**, and their related aryl-naphthalene lignans.

Received: June 26, 2022

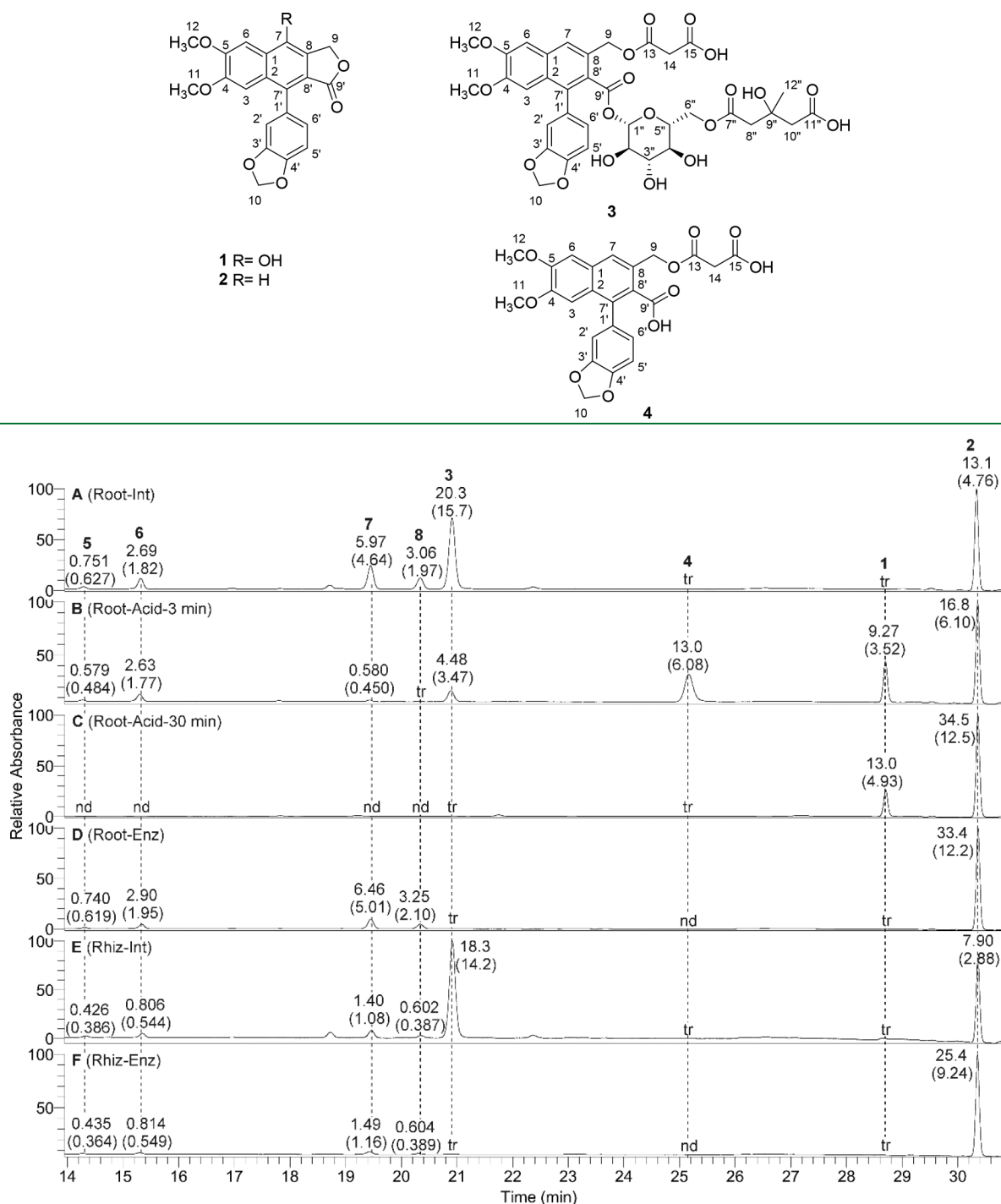


Figure 1. HPLC-UV ($\lambda = 230\text{--}400$ nm, total scan) chromatograms of the *L. austriacum* root (A–D) and rhizome (E and F) extracts, using the gradient elution 1. Intact root (A, Root-Int) and rhizome (E, Rhiz-Int) samples, their enzyme treated counterparts (D, Root-Enz and F, Rhiz-Enz, respectively), and root extracts after 3 min (B, Root-Acid-3 min) and 30 min (C, Root-Acid-30 min) of acid treatments (in 2 M trifluoroacetic acid at 100°C) were analyzed. Amounts of compounds in the dried samples, expressed in $\mu\text{mol/g}$ (printed in normal) and mg/g (printed in parentheses) values, are also indicated (tr, trace amounts are below calibration range; nd, not detected). Intact root and rhizome samples correspond to those collected in September that are marked with A1 in [Supporting Table S2](#). The amounts of compounds in the intact and enzyme-treated root and rhizome samples are the averages of three separate experiments, and the differences characterized by the relative standard deviation (RSD) values were less than 4.9%.

Arylnaphthalene lignans have also been identified in some common *Linum* (flax) species, such as in *Linum austriacum* L. and *Linum perenne* L.¹³ These two blue-flowered flax species are closely related and similar but can be distinguished from each other based on their deflexed (*L. austriacum*) or erect (*L. austriacum*) mature fruit pedicels.¹⁴ Both are native to Eurasia;

however, as ornamental, *L. perenne* is also cultivated worldwide.¹⁵ These perennial plants develop a woody, underground rhizome that bears roots and flowering leafy shoots ([Supporting Figure S1](#)).¹⁴ The phytochemical compositions of the aerial parts and various *in vitro* explants of these two flax species have been previously analyzed. Compound 2 was the main

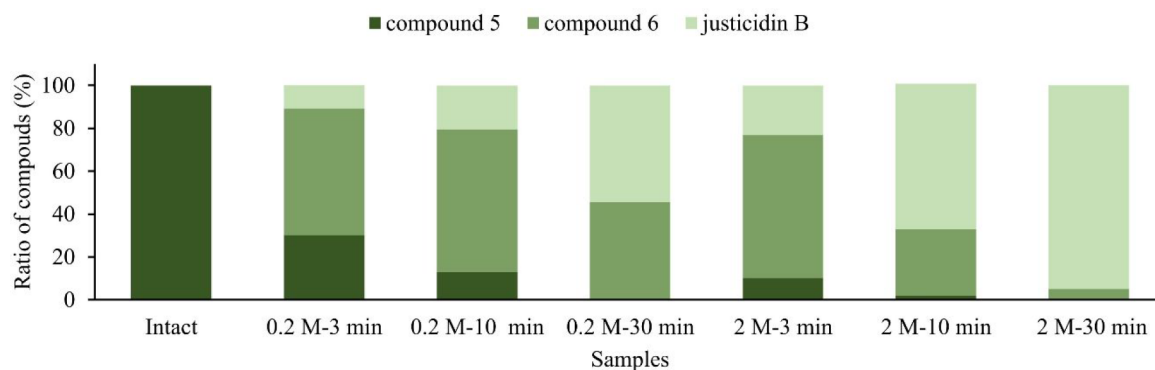


Figure 2. Effect of acid treatment on the aliquots of isolated compound 3 (Intact) that were heated at 100 °C for 3, 10, and 30 min with 0.2 M (0.2 M-3 min, 0.2 M-10 min, and 0.2 M-30 min) and 2 M trifluoroacetic acid (2 M-3 min, 2 M-10 min, and 2 M-30 min). Amounts of compounds are expressed in percentages (%), calculated from the μmol values of compounds in the samples as follows: amount of a compound in the sample (μmol) was divided by the total amount of compounds (μmol) in the same sample $\times 100$.

arylnaphthalene lignan in all tissues, reaching its highest levels in the hairy root cultures of *L. austriacum* (17 mg/g)¹⁶ and *L. perenne* (37 mg/g).² In addition to compound 2, compound 1 and glycosides of 1 have also been identified in the aerial parts and *in vitro* cultures of *L. perenne*.^{2,13,17} However, no data are available regarding the arylnaphthalene lignan composition of rhizomes and roots of *L. austriacum* and *L. perenne* grown outdoors. Several aryltetralin lignans (e.g., podophyllotoxin and methoxypodophyllotoxin) that are structurally and biosynthetically related to arylnaphthalenes are accumulated in much higher amounts in underground organs than in aerial parts, thus highlighting the relevance of also analyzing the roots and rhizomes of *L. austriacum* and *L. perenne*.^{18,19}

The aims of our research were (i) to determine the arylnaphthalene lignan composition in the underground organs (i.e., in roots and rhizomes) of wild-grown *L. austriacum* and of *L. perenne* cultivated as ornamental, (ii) to follow arylnaphthalene lignan accumulation in the roots and rhizomes during a full vegetation cycle of one year, (iii) to develop acidic and enzymatic treatment procedures for arylnaphthalene lignan conversions, aiming to obtain structure specific information and increase levels of minor compounds, (iv) to define optimum tissues and treatment procedures, allowing for the isolation of high-purity arylnaphthalene lignans, (v) to determine the structures of the isolated compounds by MS/MS, NMR, and computational calculations, and (vi) to evaluate the *in vitro* antiviral and antiproliferative potential of the isolated arylnaphthalene lignans against the SARS-CoV-2 virus and on 10 various human cancer cell lines, respectively.

RESULTS AND DISCUSSION

Conversion Study of Compounds and Identification by High-Performance Liquid Chromatography Coupled to Ultraviolet (UV) Spectrophotometry and High-Resolution Tandem Mass Spectrometry (HPLC-HR-MS/MS). Pulverized rhizome and root tissues of *L. austriacum* were treated and extracted before their HPLC-UV-HR-MS/MS analysis with distinct procedures: Intact (not treated) tissue extracts and their counterparts prepared by acidic and endogenous enzymatic treatments were also investigated (as detailed in the Experimental Section).^{20–24} Certain enzymes, e.g., glycosidases, and their substrates are accumulated in different compartments, precluding the possibility of a reaction between enzymes and their substrates in intact plant tissues. Powdering of tissues terminates this compartmentalization, thus

allowing enzymatic conversions of many compounds in an aqueous medium.²⁴ In our previous studies, we analyzed the enzymatic conversion of lignan glycosides as a function of time, confirming their quantitative hydrolysis in under 30 min.^{20,23} Accordingly, to perform the enzymatic treatments, pulverized tissues were incubated in water at room temperature for 30 min.

The HPLC-UV-HR-MS/MS analyses confirmed the presence of six known flax lignans in the extracts prepared from the root and rhizome tissues of *L. austriacum* by various treatment procedures: compounds 1, 2, and the pentosyl-dihexoside (5), pentosyl-monohexoside (6), tripentoside (7), and dipentoside (8) of 1 (Figure 1, Supporting Table S3, and Figures S2–S12).^{13,17}

However, the molecular formulas of compounds 3 ($\text{C}_{36}\text{H}_{38}\text{O}_{19}$) and 4 ($\text{C}_{24}\text{H}_{20}\text{O}_{10}$) did not correspond to any of the metabolites already known in flax species. A conversion study was performed, aiming to provide structure-specific data that contribute to the identification of these compounds. The results of the conversions were evaluated on a quantitative basis. Comparing the chromatograms of the untreated, intact root sample (Root-Int) with its acid- (for 30 min) and enzyme-treated counterparts (Root-Acid-30 min and Root-Enz), we observed that compound 3 is unstable being converted into compound 2 (Figure 1). The increased amounts of 2 following the acidic (21.4 $\mu\text{mol/g}$) and enzymatic (20.3 $\mu\text{mol/g}$) treatments (i.e., the differences in the amount of 2 between the Root-Acid-30 min and Root-Int samples and between the Root-Enz and Root-Int samples) correspond to the amount of compound 3 in the intact extract (20.3 $\mu\text{mol/g}$), confirming that compound 3 can be quantitatively converted into 2 under these conditions (Figure 1).

The quantitative enzymatic conversion of compound 3 into 2 was also detected in the rhizome samples (Figure 1, Rhiz-Int versus Rhiz-Enz). Compound 2 does not contain any free hydroxyl group; thus, its formation was surprising as it cannot be explained by common conversion reactions of lignans, i.e., by the acidic or enzymatic hydrolyses of glycosidic bonds.^{20–24} Moreover, a short-term heating (3 min) in an acidic medium resulted in the formation of an additional compound (4 in sample Root-Acid-3 min, Figure 1), suggesting a two-step formation of 2 via an intermediate product (compound 4). Compound 4 was also found in intact tissues but only in trace amounts.

Therefore, the effects of the acid treatments on compound 3 were more extensively studied, aiming to confirm this two-step

Table 1. NMR Spectroscopic Data (600 MHz, DMSO-*d*₆) for Justicidin B (2) and Linadiacins A (3) and B (4)

position	linadiacin A (3)		linadiacin B (4)		justicidin B (2)	
	δ_{C} , type	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
1	129.33/129.28, C		128.63		132.92	
2	126.70/126.66, C		126.92		127.76	
3	104.84/104.79, CH	6.89, s	104.73	6.81, s	104.99	7.00, s
4	150.04/150.02, C		149.91		149.73	
5	150.37, C		149.97		151.45	
6	106.80, CH	7.409/7.404, s	106.80	7.38, s	106.63	7.51, s
7	125.79/125.76, CH	7.88, s	125.84	7.86, s	118.77	7.94, s
8	127.61/127.56, C		127.08		139.84	
9	64.38/64.31, CH ₂	5.29/5.26 5.27/5.24, d (13.1)	64.61	5.27, s	68.01	5.44, d (1.2)
10	101.01, CH ₂	6.10/6.07/6.06/6.01, d (1.0)	101.12	6.13/6.09, s	101.14	6.13, s
11	55.16/55.13, CH ₃	3.66/3.65, s	55.12	3.64, s	55.17	3.67, s
12	55.60, CH ₃	3.92, s	55.57	3.90, s	55.79	3.95, s
13	166.77 br, C		166.93 brd			
14	41.81 br, CH ₂	3.41, s	41.87 brd	3.40, s		
15	168.15 br, C		167.95 brd			
1'	130.54/130.47, C		131.40		128.32	
2'	110.24/110.19, CH	6.88/6.87, d (1.7)	110.38	6.87, d (1.7)	110.67	6.93, d (1.6)
3'	147.21/146.77, C		146.94		146.93 ^b	
4'	146.93/146.81, C		146.66		146.95 ^b	
5'	108.06/108.38, CH	6.96/6.89, d (8.0)	108.14	7.03, d (7.9)	107.97	7.05, d (7.9)
6'	123.59/123.47, CH	6.77/6.78 dd, (8.0, 1.7)	123.45	6.77, dd (7.9, 1.7)	123.45	6.81, dd (7.9, 1.7)
7'	136.48, C		134.80		138.33	
8'	128.08/127.92, C		130.94		117.87	
9'	166.61/166.58, C		169.89 brd		169.41	
1''	94.52/94.51, CH	5.37/5.33, d (8.2)				
2''	72.05, CH	3.09/3.08, t (8.7)				
3''	75.93/75.92, CH	3.26/3.25, t (9.0)				
4''	69.49/69.65, CH	3.11/3.08, t (9.3)				
5''	74.37/74.33, CH	3.45/3.43, m				
6''	63.21/63.43, CH ₂	4.27/4.26, dd (11.7, 1.9) 3.95/3.92, dd (11.7, 5.9)				
7''	170.54/170.52, C					
8''	45.41/45.38, CH ₂	2.66, d (14.3) 2.58/2.57, (14.3)				
9''	68.96/68.95, C					
10''	45.52/45.42, CH ₂	2.52 ^a 2.48 ^a				
11''	172.58 br, C					
12''	27.29/27.23, CH ₃	1.28/1.27, s				

^aOverlapped with the solvent signal. ^bInterchangeable signals.

conversion and to prepare compound 4 at the highest yield possible, allowing its isolation. Aliquots of compound 3 (1.00 μmol) were therefore heated at 100 °C in 0.2 and 2 M TFA for various periods (3, 10, and 30 min) (Figure 2). The results showed a relatively fast conversion of compound 3 into compound 4 that was followed by a slower decomposition of compound 4, forming 2 as the end product of this two-step reaction (Figure 2). The total amounts of the conversion products (compounds 4 and 2) and the unconverted compound 3 in the samples heated in 0.2 and 2 M TFA for various periods varied between 0.950 and 1.06 μmol . These values are comparable within the experimental error of our HPLC-UV quantitation (Figure 1) with the starting amount (1.00 μmol) of compound 3 used in this study, confirming the two-step conversion of compound 3 into compounds 4 and 2 as a quantitative process without any degradation. Because relatively high amounts of compound 4 were detected in the sample

heated with 2 M TFA for 3 min and 0.2 M TFA for 3 and 10 min (Figure 2), these treatment conditions can be used to prepare this compound before its preparative HPLC isolation. The conversion of compound 3 into 2 via intermediate 4 suggests they have closely related structures containing the same basic skeleton.

The glycosides of 1 (compounds 5–8), as expected,^{20–23} could be hydrolyzed by the acid treatment (in 2 M TFA at 100 °C), forming the aglycone 1 (Figure 1). This conversion was also a quantitative process, as the yield of 1 in the sample heated in the acidic media for 30 min (Root-Acid-30 min, 13.0 $\mu\text{mol/g}$) correlated with the total amount of glycosides (5–8) in the intact extract (Root-Int, 5: 0.751 $\mu\text{mol/g}$ + 6: 2.69 $\mu\text{mol/g}$ + 7: 5.97 $\mu\text{mol/g}$ + 8: 3.06 $\mu\text{mol/g}$ = 12.5 $\mu\text{mol/g}$, Figure 1). Because the amount of 1 was negligible in the intact sample, its isolation could only be performed after this quantitative acid hydrolysis. Despite the fact that many lignan glycosides can be

hydrolyzed by endogenous glycosidases,^{20–23} the glycosides of **1** were stable during the enzymatic treatments (Figure 1). However, as detailed above, a quantitative enzymatic conversion of compound **3** into compound **2** could be confirmed in the root and rhizome samples (Figure 1). Comparing the impact of the enzymatic and acidic treatment conditions on the formation of compound **2** from compound **3**, we can state that compound **4**, which was generated as an intermediate product during the acid treatment, was not formed by the enzymatic processes even when using shorter treatment times.

Isolation of Compounds from Selected Tissues. The metabolite composition in the underground organs (i.e., in roots and rhizomes) of wild-grown *L. austriacum* and of *L. perenne* cultivated as ornamental was analyzed during a full vegetation cycle of one year by HPLC-UV-HR-MS. All extracts showed identical metabolite compositions, containing glycosides of **1** (5–8), free **1**, and **2** as well as the unknown compounds (**3** and **4**) (Supporting Table S2). In each sample, compound **3** was measured in the highest amounts, while compounds **4** and **1** were detected only in traces. Based on these results and considering the conversion characteristics of glycosides (5–8) and compound **3** in the acidic media described above, one of the root samples containing relatively high amounts of glycosides (5–8) and compound **3** (Supporting Table S2, *L. austriacum* root sample A1, collected in September) was selected for isolation purposes (composition of this root sample is also shown in Figure 1A, chromatogram Root-Int). The extracts of this root tissue were heated in 2 M TFA for 3 or 30 min as optimum conditions determined above to prepare the highest amounts of compound **4** or of compounds **1** and **2**. The isolation of compound **4** after the 3 min acid treatment and of compounds **1** and **2** after the 30 min acid treatment can easily be performed by preparative HPLC. Starting with these isolated compounds, their structural confirmation as well as their antiviral and antiproliferative studies can also be performed.

Compound **3** was isolated from the untreated root by preparative HPLC. The purity of the isolated compound **3** was analyzed by HPLC-UV-MS, confirming that this isolate also contained 19.0% (w/w) compound **8** (Supporting Figure S13). Unfortunately, these compounds (**3** and **8**) could not be effectively separated by our preparative HPLC technique even after a method development process aiming for their baseline separation. Comparing the amounts of compounds **3** and **8** in the root and rhizome samples analyzed by HPLC-UV-MS, we could state that the quantitative ratios between these compounds were higher in the rhizomes than in the roots (ratios were calculated by dividing levels of **3** by levels of **8**, resulting in average ratio values of 32.5 and 8.4 in the rhizomes and roots, respectively (Supporting Table S2)). Theoretically, tissues in which this ratio is higher allow compound **3** to be isolated more easily with lower amounts of impurity compound **8**. The rhizome of *L. austriacum* (sample A1, collected in September) was selected to isolate compound **3**, as the quantitative ratio between compounds **3** and **8** in this sample (ratio of 34.6) represents a near-average value among rhizomes (composition of this rhizome is also shown in Figure 1E, chromatogram Rhiz-Int). Thus, compound **3** could be isolated from these rhizome samples by preparative HPLC with 96.9% purity (Supporting Figure S13), allowing its structure to be determined and its antiviral and antiproliferative potential to be tested.

Identification of Compounds with Nuclear Magnetic Resonance (NMR) Spectroscopy. The NMR spectra of

compound **2** identified as justicidin B by HPLC-HR-MS were recorded in CDCl₃ and DMSO-*d*₆, respectively (Table 1, Supporting Tables S4 and S5, and Figures S14–S22).

The ¹H and ¹³C NMR assignments of compound **2** in CDCl₃ (Supporting Table S4) were in excellent agreement with previous data of justicidin B, thereby unequivocally confirming the identity of compound **2** as justicidin B.²⁵

The unknown compounds **3** and **4** were identified by NMR analysis (Table 1, Supporting Tables S6–S8, and Figures S23–S37). The ¹H NMR spectra of **2** and compound **4** recorded in DMSO-*d*₆ are comparable, showing only one singlet signal in the spectrum of compound **4** at 3.40 ppm that has no counterpart in the spectrum of **2** (Table 1 and Supporting Figures S18 and S23). Based on the HSQC spectrum, this singlet signal belongs to a methylene group of compound **4** (C14 in Table 1, Supporting Figure S26) and shows correlations to two carbonyl signals at 166.93 and 167.95 ppm in the HMBC spectrum (Supporting Figure S27 and Table S6). One of the carbonyl carbons (166.93 ppm) also correlates with another methylene signal at 5.27 ppm. The ¹H signal of **2** (5.44 ppm), which corresponds to this methylene signal of compound **4** (5.27 ppm), was assigned to H9 in the lactone ring of **2**. The HMBC correlation between H9 and C9' could only be detected in **2** (Supporting Figures S22 and S27 and Tables S5 and S6). Consequently, the γ -butyrolactone ring of **2** is in compound **4** in an opened form esterified by malonic acid. The ¹H and ¹³C NMR spectra of compound **3** recorded in DMSO-*d*₆ show double sets of signals for most of the atoms (Supporting Figures S28 and S29). The ¹H NMR spectrum exhibited a doublet signal at 6.96 ppm that could be selectively integrated. The relative integral value of this signal was about 0.5 compared to a broader singlet at 7.88 ppm, which was chosen to be 1.0. The integral value of a group of signals between 6.86 and 6.91 ppm was about 2.5 (Supporting Figure S28). These observations suggest that compound **3** is a mixture of two isomers present in a ratio of nearly 1:1. To examine the isomers of compound **3**, ¹H NMR spectra were also recorded in DMSO-*d*₆ at different temperatures. The double sets of signals showed no substantial broadening up to 90 °C, suggesting that no conversions take place between the isomers even at higher temperatures. The 1D TOCSY experiment performed by selective excitation of the 6.96 ppm doublet revealed the hydrogens of the spin system that correspond to the aromatic spin system of the benzodioxole part (H2', H5', and H6') of **2** and compound **4** (Supporting Figure S33). The additional aromatic signals between 6.75 and 6.91 ppm show the same pattern with the same coupling values, composing another benzodioxole ring (Supporting Figure S34). The H3 signal of the naphthalene ring could also be observed in this chemical shift region, demonstrating the existence of two isomers of compound **3**. Each further signal and correlation of compound **4** was also found in the NMR spectra of compound **3**. However, the NMR spectra of compound **3** show 14 extra aliphatic carbon-bound ¹H signals relative to those of compound **4**. These signals correspond to one –CH₃, three –CH₂, and five –CH groups in compound **3** (Supporting Figures S28 and S31). The C9' carbonyl carbon, which has a weak, four-bond C–H correlation to the H7 hydrogen in compounds **3** and **4**, also exhibited a strong three-bond correlation to the doublet hydrogens at 5.33 and 5.37 ppm (H1'') in compound **3** (Supporting Figures S32 and S38 and Table S7). The chemical shift of C9' in compound **3** was shielded of about 3 ppm as a result of esterification. Performing 1D TOCSY experiments by the selective excitation of each aforementioned doublet revealed

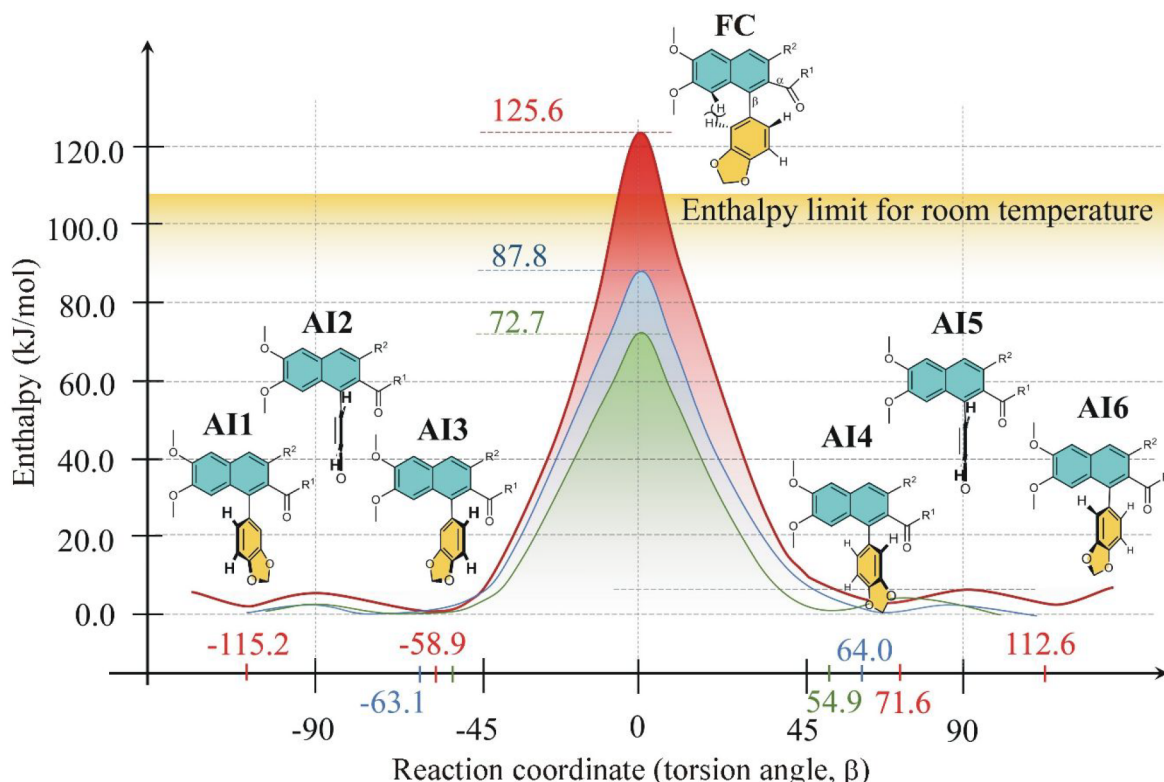


Figure 3. Computed enthalpy levels for the conformers of compound 3 (red line, with an energy maximum of 125.6 kJ/mol), compound 4 (blue line, with an energy maximum of 87.8 kJ/mol), and compound 2 (green line, with an energy maximum of 72.7 kJ/mol) as a function of rotation around the C1'–C7' bond, along with the structures of the atropisomers (AI1–AI6 and FC) representing energy minima and maxima.

that both isomers of compound 3 contain a β -glucose unit (Table 1 and Supporting Figures S35–S37). The H6'' hydrogens of the glucose show a strong correlation to the 170.5 ppm carbonyl carbon (C7'') signal, which also correlates with the doublets of a $-\text{CH}_2$ group at 2.66 and 2.57/2.58 ppm (C8''). Further HMBC correlations (Supporting Figure S38) confirmed the presence of a 3-hydroxy-3-methylglutaryl (HMG) unit in both isomers of compound 3.

The configuration of the C9'' carbon in 3 could not be determined by NMR. However, it is well-known that naturally occurring HMG esters are biosynthesized via coupling of the basic structures to (S)-HMG-CoA, resulting in the (S) configuration in their HMG unit.^{26,27}

Interestingly, the C14 and H14 signals of compounds 3 and 4 could not be detected in the NMR spectra recorded in methanol- d_4 . The lack of H14 can be explained by the hydrogen/deuterium exchange process, while the disappearance of C14 may be due to the broadening of this signal, which is also observed in DMSO- d_6 (Supporting Table S8). We concluded that both isomers of compound 3 have the same constitution, showing very small chemical shift differences in their ^1H and ^{13}C NMR spectra. However, slightly higher shifts can be detected in some signals of the benzodioxole ring and the glucose unit.

The absolute configuration of glucose in compound 3 was determined by the method of Inagaki et al.²⁸ After acidic hydrolysis of compound 3, glucose was isolated, dissolved in pyridine- d_5 , and reacted with L-cysteine methyl ester hydrochloride. The ^1H NMR spectrum of the thiazolidine derivative of the isolated glucose showed the same two characteristic signals at 5.77 and 5.51 ppm as the ^1H NMR spectrum of the thiazolidine derivative of the standard D-glucose (Supporting

Figure S39). This experiment confirmed that compound 3 contains D-glucose in its structure.

Based on these results, compounds 3 and 4 are new natural aryl-naphthalene lignan derivatives, denominated linadiacin A and linadiacin B, respectively. To determine the isomeric structures of 3, a molecular modeling study was also performed.

Computational Modeling of the Isomerization Processes. Theoretically, the two planar aromatic systems of our lignans, i.e., the benzodioxole and the naphthalene units, can rotate around the C1'–C7' bond connecting them, resulting in a changing β dihedral angle. If this rotation is hindered, special stereoisomers (atropisomers) can be distinguished.

Based on the NMR data, compound 3 is a mixture of these two atropisomers existing in an almost equal ratio; however, its structurally related aryl-naphthalene lignans 4 and 2 have no isomeric analogues. The enthalpy levels of these compounds were calculated during a full (360°) rotation of the benzodioxole unit around the C1'–C7' bond in this computational study (Figure 3). Two low-energy isomer populations, both comprising three atropisomers, could be discriminated (isomers marked with AI1–AI3 and AI4–AI6 in Figure 3). The computed enthalpy maximum for 3 is 125.6 kJ/mol, which corresponds to a conformer of 3 containing the two planar aromatic systems in the same plane (flat conformer marked with FC in Figure 3). Because the general limit for reactions at room temperature is around 100 kJ/mol, the computed enthalpy maximum (125.6 kJ/mol) is a relevant energy barrier between the two isomer populations representing low energy, thus confirming the coexistence of two atropisomer populations of compound 3.

However, members within the same population can be freely converted into each other. These calculations confirm the NMR

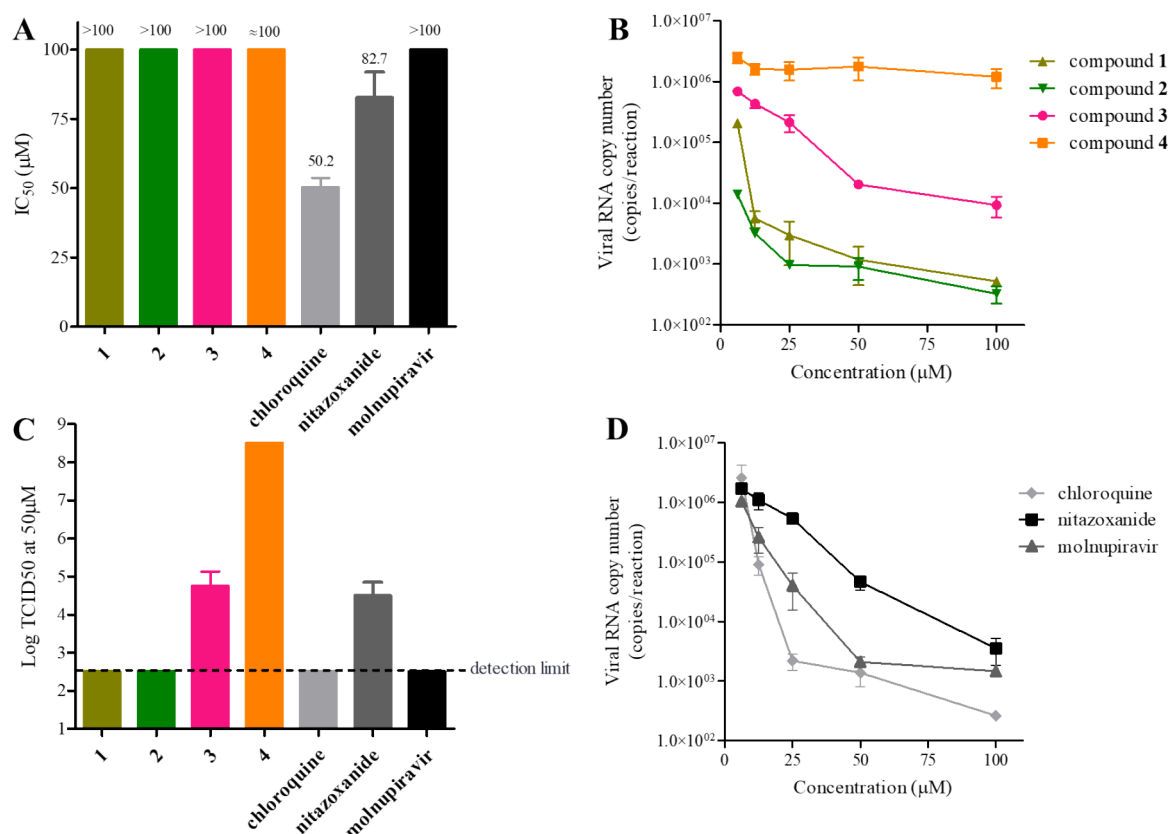


Figure 4. *In vitro* cytotoxic (A) and antiviral (B, C, and D) activity of the isolated compounds (1–4) and standard compounds (chloroquine, nitazoxanide, and molnupiravir) against the noncancer host cells Vero E6 and Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), respectively. The IC₅₀ values are compound concentrations required for 50% inhibition of the host cell viability (A). Antiviral effect was enumerated as viral copy numbers (B and D) and median tissue culture infectious doses (TCID₅₀) (C). TCID₅₀ values are dilutions of the virus required to infect 50% of the host cells.

results identifying two isomers of compound 3. The energy barriers between the atropisomers of compounds 4 and 2 were calculated to be 87 and 72 kJ/mol, respectively, allowing conversions between isomers of both compounds. These computational results explain why the isomers of 4 and 2 cannot be detected by NMR.

Mass Fragmentation Study of New Compounds 3 and 4. Mass fragmentations of compounds 3 and 4, performed by HPLC-HR-MS/MS using negative and positive ionization modes, confirmed their new structures. The mass fragmentation of the deprotonated 3 (m/z 773) (Supporting Table S9 and Figure S7) resulted in the consecutive elimination of hydroxymethylglutaryl, glucosyl, and malonyl groups (x_1 , x_2 , and x_3 in Supporting Scheme S1), forming the final product ion m/z 381 (Supporting Scheme S1 and Table S9). The formation of this ion (m/z 381) was also confirmed during the fragmentation of the deprotonated 4 (m/z 467), providing evidence of the existence of the malonyl unit in compounds 3 and 4 (Supporting Table S9 and Figures S7 and S9).

Using the positive ionization mode, compounds 3 and 4 exhibited a particularly abundant fragment ion signal at m/z 365 in their fragment ion spectra (Supporting Table S9 and Figures S6 and S8). The ion m/z 365 generated from both compounds may correspond to the protonated 2. A high-intensity signal of this ion m/z 365 can also be observed in the MS (MS1) spectra of compounds 3 and 4 due to the in-source fragmentation of these compounds (Supporting Table S9). The MS2 spectra of the protonated 2 and those of the ion m/z 365 formed from 3

and 4 were highly comparable, containing identical fragment ions (Supporting Figures S11 and S12). Accordingly, the fragment ion m/z 365 can be identified as compound 2 in the mass spectra of both new compounds (3 and 4), confirming their identical basic skeleton, which can be converted by hydrolysis and subsequent lactone formation into compound 2.

The *In Vitro* SARS-CoV-2 Virus Inhibitory Effect of the Compounds. The isolated compounds 1–4 were evaluated for their *in vitro* anti-SARS-CoV-2 potential using the noncancerous Vero E6 cells as hosts. Because our lignans are well tolerated by the Vero E6 cells (IC₅₀ > 100 μM for all lignans), these cells can be used as hosts to evaluate their SARS-CoV-2 virus inhibitory potential intracellularly (Figure 4, panel A). As antiviral standards, chloroquine, nitazoxanide, and molnupiravir were employed.^{29–31} Chloroquine (IC₅₀, 50.2 μM) and nitazoxanide (IC₅₀, 82.7 μM) were cytotoxic on Vero E6 cells, while molnupiravir (IC₅₀ > 100 μM) was not (Figure 4, panel A).

The dose-dependent activity of compounds 1–3 and the inactivity of 4 against the SARS-CoV-2 virus were measured in the concentration range from 6.25 to 100 μM by quantifying the viral RNA copy number (Figure 4, panel B). Compound 2 was the most effective, followed by 1. Both compounds showed a greater than 3-log reduction in viable viruses at a treatment concentration as low as 12.5 μM. This antiviral activity was higher than that of the standard drugs (Figure 4, panels B and D). The antiviral activity of the isolated compounds and standards was further confirmed by their TCID₅₀ values (Figure 4, panel C). Our results are in good accordance with those of a

Table 2. Antiproliferative Effect of the Isolated Compounds against Human Lung Cancer Cell Lines (A549, Calu-1, EBC-1, and H838), as Well as Human Melanoma (A2058), Prostate Cancer (PC-3), Hepatoblastoma (HepG2), Glioma (U87), Colorectal Carcinoma (HT-29), and Monocytic Leukemia (MM6) Cells

cell lines	compounds				
	3	4	1	2	etoposide
	IC ₅₀ (μM) ^a				
A549	26.3 ± 3.4	>100.0	8.8 ± 1.7	11.4 ± 1.2	>100.0
Calu-1	67.0 ± 10.2	>100.0	31.8 ± 3.8	18.5 ± 3.6	>100.0
Ebc-1	19.2 ± 7.5	16.0 ± 2.5	14.8 ± 3.1	63.2 ± 5.7	15.7 ± 4.6
H838	16.0 ± 2.7	>100.0	16.0 ± 2.5	11.0 ± 2.0	>100.0
A2058	2.4 ± 0.4	25.1 ± 2.5	2.6 ± 0.4	8.9 ± 2.2	8.9 ± 1.9
PC-3	3.2 ± 0.4	32.8 ± 4.9	3.8 ± 0.2	5.7 ± 1.0	8.0 ± 1.9
HepG2	>100.0	>100.0	21.6 ± 2.7	16.8 ± 1.8	20.9 ± 3.2
U87	>100.0	>100.0	26.1 ± 12.2	21.6 ± 4.6	27.0 ± 8.3
HT-29	19.9 ± 0.0 ^b	>100.0	19.5 ± 0.1	5.9 ± 0.1	18.5 ± 3.6
MM6	7.1 ± 1.2	70.1 ± 5.7	3.9 ± 0.9	2.5 ± 1.1	2.2 ± 1.2

^aResults are presented as means ± SD, calculated from four parallel tests performed two times independently. ^bCalculated SD value in this case is 0.04.

very recent study that confirmed the anti-SARS-CoV-2 activity (IC₅₀, 1.92 μM) and low cytotoxicity (IC₅₀ value >100 μM) in the Vero E6 host cells for **1**.⁸

Literature results also show that the antiviral activity of the monoglycoside of **1** against SARS-CoV-2⁸ and tick-borne encephalitis virus (TBEV)⁹ as well as that of the diglycoside and triglycoside of **1** against the human immunodeficiency virus 1 (HIV-1)⁵ was 3.4, 6.3, 13.0, and 62.1 times lower than the antiviral activity of the free aglycone **1**, respectively. Due to these results, glycosides of **1** (compounds **5**–**8**) were not isolated from our *Linum* samples to test their anti-SARS-CoV-2 activity. Comparing the antiviral activity and polarity of *Linum* lignans, it can be concluded that the increasing polarity of these compounds (glycosides of **1** > **3** > **4** > **1** > **2**) results in a decrease in their activity.

Antiproliferative Activity of the Isolated Compounds.

The antiproliferative activity of the isolated compounds was studied on 10 various human tumorous cell cultures, i.e., four lung-originated cancer cell lines (A549, Calu-1, EBC-1, and H838), one melanoma (A2058), prostate (PC-3), hepatoblastoma (HepG2), glioma (U87), colorectal carcinoma (HT-29), and monocytic leukemia (MM6) cancer cell line. The aryltetralin lignan derivative etoposide was used in the *in vitro* cytostatic assay as a standard compound.³² Recently, the antiproliferative activity of **1** against A549 cells with IC₅₀ values of 2.02 μM⁵ and 24.01 μM⁴ and PC-3 cells with the IC₅₀ value of 7.2 μM³³ has been demonstrated. Our results support the antiproliferative potential of **1** against these cells by representing IC₅₀ values lower than 10 μM (Table 2). Furthermore, the activity of **1** and **2** on several cell types (A2058, PC-3, HepG2, U87, HT-29, and MM6) and the activity of **3** on A2058 and PC-3 cells were comparable to that of the etoposide.

To summarize, the underground organs, i.e., roots and rhizomes, of *L. austriacum* and *L. perenne* were confirmed as the sources of two already known aryltetralin lignans, diphyllin (**1**) and justicidin B (**2**), and two new aryltetralin lignans denominated linadiacin A (**3**) and B (**4**). In all tissue samples analyzed over a one-year vegetation cycle, **3** was the main compound, while only trace amounts of **4** and **1** were detected. Selection of tissues that contain the lowest levels of impurities interfering with the isolation, conversion of **3** into **2** via the intermediate **4**, and generation of **1** from glycosides allowed for the high-purity isolation of compounds by one-step preparative

HPLC. Compound **3** from intact rhizomes, **4**, **1**, and **2**, from root extracts heated in 2 M TFA for 3 min (compound **4**) and 30 min (compounds **1** and **2**) could be isolated.

The results of the conversion, HPLC-tandem mass spectrometry (MS/MS), nuclear magnetic resonance spectroscopy, and molecular modeling studies allowed the structure to be unambiguously identified.

The *in vitro* anti-SARS-CoV-2 activity of compound **2** was confirmed, exhibiting a greater than 3-log reduction in viable viruses at 12.5 μM concentration and low toxicity on Vero E6 host cells even at higher concentrations. Compounds **1**–**3** were also confirmed as moderately active antiproliferative compounds, expressing IC₅₀ values in the micromolar concentration range against various cancer cell lines.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotation was measured at 25 °C on a Jasco-P-200 polarimeter. Analytical HPLC with UV and high-resolution Orbitrap mass spectrometry: A Dionex Ultimate 3000 UHPLC system (3000RS diode array detector (DAD)), TCC-3000RS column thermostat, HPG-3400RS pump, SRD-3400 solvent rack degasser, WPS-3000TRS autosampler) connected to an Orbitrap Q Exactive Focus Mass Spectrometer equipped with electrospray ionization (ESI) (Thermo Fisher Scientific, Waltham, MA) was used. Column: Kinetex C18 column (75 × 3 mm; 2.6 μm) (Phenomenex, Torrance, CA). Eluents: eluent A, 0.1% v/v formic acid; eluent B, acetonitrile:0.1% v/v formic acid (80:20, v/v). Gradient program 1:0.0 min, 20% B; 24.0 min, 40% B (linear gradient); 30.0 min, 70% B (linear gradient); 31.0 min, 70% B (isocratic); gradient program 2:0.0 min, 20% B; 10.0 min, 70% B (linear gradient); 12.0 min, 90% B (linear gradient). Flow rate: 0.3 mL/min; column temperature: 25 °C; injected volume: 1.0–10.0 μL. The ESI source was operated in the positive and negative ionization mode (switching mode), and operation parameters were optimized automatically using the built-in software. Working parameters: scan rate, about 0.56 scan/s; spray voltage, 3500 V (+), 2500 V (−); capillary temperature 256 °C; sheath-, auxiliary-, and spare-gases (N₂): 47.50, 11.25, and 2.25 arbitrary units, respectively. The resolution of the full scan was 70 000, and the scanning range was between 100 and 1000 *m/z* units. MS/MS scans were acquired at a resolution of 35 000 using a collision energy of 10, 20, and 30 eV. DAD spectra were recorded between 230 and 400 nm.

To quantify the compounds, an external standard method was applied using UV (total scan) chromatograms detected in the wavelength range of 230–400 nm. Linear regression analyses of the isolated compounds (**1**–**4**) were performed in the range of 0.888–200.0 ng of their injected amounts, resulting in the appropriate *r*² values

(higher than 0.9998 for each compound) (Supporting Figure S40). The amounts of glycosides (compounds 5–8) were calculated using the calibration curve of their isolated aglycone (compound 1).

Preparative HPLC. A Pharmacia LKB HPLC (Uppsala, Sweden) system (2248 pumps, VWM 2141 UV detector) was connected to a preparative HPLC column: Gemini NX-C18 (5 μ m), 25 $\times$ 1 cm (Phenomenex, Torrance, CA). The eluents were the same as described above. Gradient: 0.0 min, 20% B; 50.0 min, 50% B (linear gradient); 55.0 min, 70% B (linear gradient); 90 min, 70% B (isocratic); flow rate: 5.0 mL/min; column temperature: ambient; injected volume: 500 μ L.

Nuclear Magnetic Resonance (NMR) Spectroscopy. NMR spectra of compounds 2–4 were recorded at 25 $^{\circ}$ C on a Varian DDR spectrometer (599.9 MHz for 1 H and 150.9 MHz for 13 C) equipped with a dual 5 mm inverse detection gradient (IDPFG) probe-head. NMR spectra of all the isolated compounds were recorded in DMSO- d_6 ; in addition, NMR spectra of compound 2 were also recorded in $CDCl_3$ and of compounds 3 and 4 in methanol- d_4 . Standard pulse sequences and parameters were used to obtain 1D 1 H, 1D 13 C, 2D [1 H– 1 H] COSY, [1 H– 13 C] HSQC, and [1 H– 13 C] HMBC spectra. For compound 3, 1D TOCSY and band selective HMBC (bsHMBC) experiments were also performed. 1D TOCSY-s spectra were acquired by selective excitation of the 1 H signals at 5.33, 5.37, and 6.97 ppm using various mixing times between 0 and 120 ms. bsHMBC-s experiments were performed by selective excitation of the 123–133, 145–152, and 165–175 ppm 13 C chemical shift ranges. 1 H and 13 C chemical shifts were referenced relative to the solvent resonances.

1 H NMR spectra for the determination of the optical activity of glucose were recorded in pyridine- d_5 at 18 $^{\circ}$ C on a Varian Mercury Plus spectrometer (400.0 MHz for 1 H) and referenced to the solvent signal.

Ultraviolet Spectroscopy. $\lambda_{\max}$ and specific absorbance values ($[A]_{1\text{cm}}^{1\%}$) of compounds 3 and 4 were determined in MeOH by a Jasco V-550 UV/vis spectrophotometer.

Computational Method. All computations were carried out with the Gaussian16 program package (Gaussian Inc., Wallingford, CT, 2016)³⁴ as described in our previous publication.³⁵

The materials and reagents applied in the analysis and isolation of plant metabolites, such as acetonitrile (ACN), D-glucose, distilled water, formic acid, methanol, trifluoroacetic acid (TFA) (Reanal, Hungary), $CDCl_3$, $CHCl_3$, DMSO- d_6 , methanol- d_4 , pyridine- d_5 (VWR Chemicals, Belgium), and L-cysteine methyl ester hydrochloride (Sigma-Aldrich, St. Louis, MO) were all of analytical reagent grade of the highest purity available.

Plant Material. Underground organs, i.e., roots and rhizomes, of *Linum austriacum* L. and *L. perenne* L. (Linaceae) were collected from different Hungarian locations four times during a full vegetation cycle of one year: in April, June, September, and December of 2020. Sample collection locations of *L. austriacum* were near Szólád village (GPS, 46.783695, 17.878119), near Szarvaskő village (GPS, 47.983711, 20.334509), and near Budapest (47.496522, 18.973499) (marked with A1, A2, and A3, respectively), which are known habitats of this plant. Collection locations of *L. perenne* grown as an ornamental plant were in Budapest (GPS, 47.499692, 19.008675) and in the village Nágocs (GPS, 46.658458, 17.960496) (marked with P1, and P2, respectively). At each sampling time, at least 10 plants were collected. The roots and rhizomes of these plants were manually separated. These roots and rhizomes were pooled to prepare the rhizome and root samples.

The rhizome and root samples were lyophilized on the day of collection. The voucher specimens of the dried samples are deposited in the Department of Plant Anatomy, Eötvös Loránd University, Budapest, Hungary.

Performing Endogenous Enzymatic Treatment. Lyophilized and pulverized plant tissues (100.0 mg) were suspended in 2.0 mL of distilled water in 25 mL screw-capped vials. These suspensions were left for 30 min at room temperature, allowing for enzymatic conversions of compounds in an aqueous medium. Thereafter, the samples were lyophilized and extracted according to the protocol shown in the following section.

Preparation of Plant Extracts for Analysis and Isolation. Lyophilized and pulverized intact plant tissues (100.0 mg), as well as enzyme-treated and lyophilized tissues, were extracted three times

consecutively with 5 mL of methanol at 60 $^{\circ}$ C in 25 mL screw-capped vials for 30 min to prepare 15.0 mL stock solutions. These stock solutions were used for HPLC-UV-HR-MS analyses. Dried aliquots (5.0 mL) of the stock solutions prepared from the intact *L. austriacum* root and rhizome samples (collected in September and marked with collection number A1) were dissolved in 0.5 mL of methanol for the isolation of 3 by preparative HPLC.

Performing Acid Treatments. Acid treatment optimization of isolated 3 for the production of 4: 0.774 mg of isolated 3 (corresponding to 1.00 μ mol) was dissolved in 1.0 mL of 0.2 M TFA and 2 M TFA (in 5 mL screw-capped vials). These solutions were heated for 3, 10, and 30 min at 100 $^{\circ}$ C. The samples were dried using a rotary vacuum evaporator (at 30–40 $^{\circ}$ C). The dried samples were analyzed after their dissolution in methanol by HPLC-UV-HR-MS.

Hydrolysis of 3 to release the glucose unit: 10.0 mg of 3 was dissolved in 10 mL of 2 M TFA (in 50 mL round-bottom flask) and heated under reflux (at 100 $^{\circ}$ C) for 120 min. The sample was dried using a rotary vacuum evaporator (at 30–40 $^{\circ}$ C).

Acid treatments of root and rhizome extracts: Dried aliquots (5.00 mL) of the stock solution prepared from the intact *L. austriacum* root sample (collected in September and marked with collection number A1) were dissolved in 1.0 mL of 2 M TFA (in 5 mL screw-capped vials). These solutions were heated for 3 and 30 min at 100 $^{\circ}$ C. After heating, the samples were dried using a vacuum evaporator (at 30–40 $^{\circ}$ C). The dried samples were dissolved in 5.00 and 0.5 mL of methanol before the analyses and isolations, performed by HPLC-UV-HR-MS and preparative HPLC, respectively. The isolation of 4 after the 3 min acid treatment and of 1 and 2 after the 30 min acid treatment was performed by preparative HPLC.

Linadiacin A (3). Colorless, amorphous solid; $[\alpha]_D^{25} = 28.1$ (c 0.1, MeOH); UV (MeOH) $\lambda_{\max} = 257.7$ nm; $[A]_{1\text{cm}}^{1\%} = 487.6$; for 1 H NMR and 13 C NMR data, see Table 2; HRESIMS m/z 773.1930 $[M - H]^-$ (calcd for 773.1924, $C_{36}H_{37}O_{19}$); for all HR-Orbitrap-MS data, see Supporting Table S3.

Linadiacin B (4). Colorless, amorphous solid; UV (MeOH) $\lambda_{\max} = 246.1$ nm; $[A]_{1\text{cm}}^{1\%} = 872.1$; for 1 H NMR and 13 C NMR data, see Table 2; HRESIMS m/z 467.0978 $[M - H]^-$ (calcd for 467.0973, $C_{24}H_{19}O_{10}$); for all HR-Orbitrap-MS data, see Supporting Table S3.

Determination of the Absolute Configuration of Glucose.

Glucose was released from compound 3 by acidic hydrolysis (as detailed above). The hydrolyzed, dried sample containing glucose was dissolved in 5.0 mL of distilled water. The formed suspension was centrifuged to remove insoluble precipitate. The supernatant was dried (at 30–40 $^{\circ}$ C, using a rotary vacuum evaporator) and redissolved in 1.0 mL of aqueous methanol (methanol:H₂O 1:1, v/v). Glucose was then isolated by thin layer chromatography (TLC) using a silica gel 60 F254 plate (Merck, Germany) and an eluent mixture of $CHCl_3$:MeOH:H₂O (8:7:2, v/v).³⁶ The spots of glucose ($R_f = 0.30$) were collected, suspended in aqueous methanol (methanol:H₂O 1:1, v/v), and dried (at 30–40 $^{\circ}$ C, using a rotary vacuum evaporator). The isolated glucose (1 mg) and L-cysteine methyl ester hydrochloride (3 mg) were dissolved in 650 μ L of pyridine- d_5 , heated at 60 $^{\circ}$ C for 1 h, and left overnight at room temperature. The thiazolidine derivative of glucose was then measured by NMR.²⁸ The thiazolidine derivative of standard D-glucose was also prepared and analyzed.

Antiviral Experiments. All infection experiments were performed under Biosafety Level-3 (BSL-3) conditions at the National Biosafety Laboratory, National Public Health Center (Budapest, Hungary).

Cells and Virus. Vero E6 host cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC 85020206) and maintained in DMEM high-glucose (4.5 g/L) medium (Lonza) containing 10% FBS (Gibco) and supplemented with 2 mM of L-glutamine (Lonza), 1 mM sodium pyruvate (Merck), and CellCultureGuard (PanReacApplichem) at 37 $^{\circ}$ C in a humidified atmosphere of 5% CO₂.

The virus was isolated from an upper respiratory specimen of a COVID-19 patient in March 2020 and passaged three times. Whole genome sequencing confirmed that the strain belongs to the original lineage of the SARS-CoV-2 (EPI_ISL_1172022). The viral titer, which

was determined by a 50% tissue culture infective dose, was 10^6 TCID₅₀/mL.

Selectivity study of the compounds on Vero E6 host cells: To evaluate the selectivity of the compounds, their cytotoxicity was determined on Vero E6 host cells using the Alamar Blue viability assay. Vero E6 cells were plated in 96-well flat-bottom tissue culture plates in 10% FBS containing DMEM media (5000 cells/100 μ L) 1 day before the experiment. Before the test, 50 μ L of supernatant was removed and replaced with a 50 μ L serum-free DMEM (SFM) medium. Compounds were serially diluted with SFM DMEM medium and added to the cells. The final concentration range was between 6.25 and 100 μ M, and the final FBS concentration was 2.5%. After 2 days, a 22 μ L Alamar Blue (resazurin sodium salt, Merck) solution (0.15 mg/mL in PBS) was added to each well, and after 4 h of incubation, the fluorescence was measured at $\lambda_{\text{Ex}} = 530/30$ and $\lambda_{\text{Em}} = 610/10$ nm using a Synergy H4 multimode microplate reader (BioTek). All measurements were performed in quadruplets, and the mean IC₅₀ values together with $\pm$ SEM were represented.

Antiviral Activity of the Compounds. Vero E6 cells were plated in 96-well flat-bottom tissue culture plates in 10% FBS containing DMEM medium (5000 cells/100 μ L) 1 day before the experiment. After removing the supernatant, Vero E6 cells were infected with the SARS-CoV-2 virus at a multiplicity of infection (MOI) of 0.05, and cells were incubated with the viruses for 1 h. Then, the supernatant was removed, and infected cells were incubated with 200 μ L of serially diluted compound solutions in DMEM-containing 5% FBS. The inoculated cultures were grown in a humidified 37 °C incubator in an atmosphere of 5% CO₂. After 48 h of incubation, 50 μ L of virus-containing supernatants was transferred to 96-well plates, and viral RNA was extracted using a PerkinElmer Chemagic Viral RNA/DNA Kit (PerkinElmer) and a PerkinElmer Chemagic automated extraction machine. RT-qPCR was conducted using the SARS-CoV-2 RT-qPCR kit (PerkinElmer) according to the manufacturer's instructions on a Roche LightCycler 480 with an automated second-derivative evaluation method. The SARS-CoV-2 RT-qPCR kit (PerkinElmer) targeted two specific genomic regions of SARS-CoV-2: the nucleocapsid (N) gene and ORF1ab. Experiments were performed in quadruplets and repeated at least two times. The assay was validated by negative (medium-treated virus control) and positive controls (chloroquine, nitazoxanide, and molnupiravir).

To calculate the TCID₅₀/mL values, VERO E6 cell monolayers in 96-well titration plates were inoculated with 10-times serially diluted SARS-CoV-2 isolate or 10 μ L of supernatants. The cell monolayers were incubated for 5 days, then fixed with 4% formaldehyde solution, washed with water, and stained with 0.5% crystal violet in PBS for 2 h at room temperature. Clear plaque numbers were visualized, and the viral titers were measured by determining the TCID₅₀/mL using TCID₅₀ calculator v2.1 [Binder M. TCID₅₀ Calculator (v2.1–20–01–2017_MB) (accessed on 10 June 2020); Available online: https://www.klinikum.uni-heidelberg.de/fileadmin/inst_hygiene/molekulare_virologie/Downloads/TCID50_calculator_v2_17-01-20_MB.xlsx]. Experiments were done in quadruplets and repeated at least two times.

Antiproliferative Activity of Compounds. Cell Culturing and Media. For the antiproliferative experiments, the following human cell lines were used: A549 (epithelial, lung carcinoma), Calu-1 (epidermoid, bronchial carcinoma, non-small cell lung cancer, Sigma 93120818), H838 (epithelial, alveolar carcinoma, non-small cell lung cancer), EBC-1 (lung squamous cell carcinoma), A2058 (melanoma, derived from metastatic site: lymph node), PC-3 (prostate adenocarcinoma), HepG2 (hepatoblastoma), U87 (glioblastoma), HT-29 (human colorectal carcinoma), and MM6 (MonoMac-6, monocytic leukemia). Calu-1 was purchased from Sigma, and the other cell lines were generous gifts from Dr. József Tóvári (Department of Experimental Pharmacology, National Institute of Oncology, Budapest, Hungary).

For maintaining cell cultures, DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 μ g/mL penicillin/streptomycin, 1 mM pyruvate, and 1% nonessential amino acids (CM DMEM) were used. A2058 cells were cultured in RPMI-1640 medium supplemented with

10% FBS, 2 mM L-glutamine, and a penicillin–streptomycin antibiotics mixture (50 IU/mL and 50 μ g/mL, respectively). The cultures were maintained at 37 °C in a humidified atmosphere with 5% CO₂. MonoMac6 and HepG2 were maintained in RPMI-1640 medium (Lonza) containing 10% FBS supplemented with 2 mM L-glutamine and 160 μ g/mL gentamicin (for MonoMac6) or 1% penicillin–streptomycin (for HepG2).

Determination of the Cytostatic Activity of the Compounds. The cells were grown to confluency and then divided into 96-well tissue culture plates with the initial cell number of 5000 cells/well. Cells were incubated at 37 °C in a 5% CO₂ humidified atmosphere. Before the test, 50 μ L of the supernatant was removed and replaced with 50 μ L of serum-free medium. Compounds were serially diluted with medium and added to the cells. The final concentration range was between 0.4 and 100 μ M, and the final FBS concentration was 2.5%. After 24 h of incubation, cells were washed 3 times with SFM and then replaced by 10% FBS containing media. Three days after, 22 μ L of Alamar Blue (resazurin sodium salt, Merck) solution (0.15 mg/mL in PBS) was added to each well, and after 4 h of incubation, the fluorescence was measured at $\lambda_{\text{Ex}} = 530/30$ and $\lambda_{\text{Em}} = 610/10$ nm using a Synergy H4 multimode microplate reader (BioTek). OD₆₁₀ values were subtracted from OD₅₃₀ values. The percent of cytostasis was calculated with the following equation: cytostatic effect (%) = $[1 - (\text{OD}_{\text{treated}}/\text{OD}_{\text{control}})] \times 100$, where the values OD_{treated} and OD_{control} correspond to the optical densities of the treated and the control wells, respectively. In each case, two independent experiments were carried out with four parallel measurements, and the mean IC₅₀ values together with $\pm$ SD were represented.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jnatprod.2c00580>.

Photo of the underground organs of *L. austriacum*; lignan composition of the underground organ samples; HR-MS, HR-MS/MS data, and spectra of 1–8; mass fragmentation route of 3 and 4; HPLC-UV chromatograms of isolated 3; NMR spectra and data of 2–4; NMR spectra of the thiazolidine derivatives of standard and isolated glucose; HPLC-UV calibration curves of 1–4 (PDF)

Data for compound 3 (ZIP)

Data for compound 4 (ZIP)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the National Research, Development and Innovation Office, Hungary (grants: EFOP-1.8.0-VEKOP-17-2017-0000 (S.B., K.H., I.B., G.M.K., B.P., and Z.K.), VEKOP-2.3.3-15-2017-00020, TKP2021-NVA-14, OTKA NKFIH K-135712, OTKA KH-130401 and OTKA K-142904 and , the János Bolyai Research Scholarship of the Hungarian Academy of Sciences (G.T. and Z.M., BO/799/21/7). The financial support from the Bolyai+ New National Excellence Program and the ÚNKP-22-ME3 project of the Ministry for Innovation and Technology is highly appreciated (G.T. and Z.M.). K.H. and S.B. are grateful for the ELTE Thematic Excellence Programme (Szint+) and the 2018-1.2.1-NKP-2018-00005 project (under the 2018-1.2.1-NKP funding scheme) provided by the Hungarian Ministry for Innovation and Technology and for the National Research, Development and Innovation Office, Hungary. K.H. is thankful for the grant provided by the Lendület (Momentum) Programme (LP2021-4) of the Hungarian Academy of Sciences.

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