Lignans Isolated from Valerian: Identification and Characterization of a New Olivil Derivative with Partial Agonistic Activity at A_1 Adenosine Receptors

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A methanolic extract of the roots of *Valeriana officinalis* (valerian) was investigated for its lignan content. In addition to the lignans 8′-hydroxypinoresinol (**1**) and pinoresinol-4-*O*-*β*-D-glucoside (**2**), which had already been isolated from valerian in an earlier study, the 7,9′-monoepoxylignans massoniresinol-4′-*O*-*β*-D-glucoside (**3**), 4′-*O*-*β*-D-glucosyl-9-*O*-(6″-deoxysaccharosyl)olivil (**4**), and berchemol-4′-*O*-*β*-D-glucoside (**5**) and the 7,9′:7′,9-diepoxylignans pinoresinol-4,4′-di-*β*-*O*-D-glucoside (**6**), 8-hydroxypinoresinol-4′-*O*-*β*-D-glucoside (**7**), and 8′-hydroxypinoresinol-4′-*O*-*β*-D-glucoside (**8**) were identified. While lignans **3**, **6**, **7**, and **8** had already been isolated from other plants, lignans **4** and **5** are new natural products. The lignans were investigated in radioligand binding assays at various receptors of the central nervous system, including $GABA_A$, benzodiazepine, $5\text{-}HT_{1A}$, and adenosine A_1 and A_{2A} receptors, to investigate their potential contribution to the pharmacological activity of valerian. The novel olivil derivative **4** proved to be a partial agonist at rat and human A_1 adenosine receptors exhibiting A_1 affinity and activity in low micromolar to submicromolar concentrations. Lignan **4** is the first nonnucleoside adenosine receptor agonist not structurally related to adenosine.

Phytopharmaceuticals are increasingly popular in many countries, including Europe and the United States. The extracts of the roots of *Valeriana officinalis* L. (valerian), Valerianaceae, belong to the most frequently used sedative herbal medicines. Valerian is applied as a tranquillizer for the treatment of nervousness and agitation and as a mild sedative for sleep promotion.[1,2] It is considered a safe medication, since it produces no or little side effects.[3] The mild CNS-depressant effects of valerian have been demonstrated in mice,[4] as well as in some clinical studies.[3,5] Although the major constituents of valerian, including valepotriates and sesquiterpenes, have been isolated and pharmacologically investigated, the molecular mechanism of the sedative action is still unclear. It has been found that constituents other than the valepotriates probably contribute to the pharmacological activity of valerian.[5,6]

Direct and indirect interaction of valerian extract with $GABA_A$ receptors has been postulated.[1,7] Balduini and Cattabeni described binding of a hydroalcoholic valerian extract to A_1 adenosine receptors, but found no interaction with the benzodiazepine binding site of $GABA_A$ receptors.[8] We have recently found that a polar extract of valerian exhibited agonistic activity at rat and human A_1 adenosine receptors.[9]

Recently, several lignans were isolated from valerian extracts.[10] One of the constituents, 8′-hydroxypinoresinol (**1**), was found to exhibit affinity to $5\text{-}HT_{1A}$ receptors in low micromolar concentrations.[10]

The present study was aimed at identifying further lignans in polar (methanolic) extracts of *V. officinalis* and investigating their pharmacological activities in vitro.

Results and Discussion

Chemistry. A methanolic extract of the roots of *V. officinalis* was prepared and fractionated by preparative reversed-phase HPLC, followed by preparative thin-layer chromatography to yield pure lignans. In addition to the earlier described lignans 8′-hydroxypinoresinol (**1**) and pinoresinol-4-*O*-*β*-D-glucoside (**2**),[10] six other lignans were isolated and characterized (Figure 1). Structure elucidation was achieved essentially by ^1H and ^{13}C NMR spectroscopy, including two-dimensional correlated NMR spectroscopy methods, and NOE difference spectroscopy. In addition, electron impact and, if necessary, fast atom bombardment or electrospray mass spectra were recorded.

Structures and ^1H and ^{13}C NMR spectral data are provided in Figure 1 and Tables 1 and 2. The lignan derivatives, which have not been previously described as constituents of valerian, were identified as the 7,9′-monoepoxylignans massoniresinol-4′-*O*-*β*-D-glucoside (**3**), 4′-*O*-*β*-D-glucosyl-9-*O*-(6″-deoxysaccharosyl)olivil (**4**), and berchemol-4′-*O*-*β*-D-glucoside (**5**). Compound **3** has been previously isolated from the roots of *Saussurea lappa*, a Chinese medicinal plant;[11] compound **4** is a new natural product, the saccharoside of olivil-4′-*O*-*β*-D-glucoside, which has been isolated from various plants;[12,13] and compound **5** is another new natural product, the aglycon of which has been isolated from *Berchemia racemosa*.[14]

The other lignans were identified as the 7,9′:7′,9-diepoxylignans pinoresinol-4,4′-di-*O*-*β*-D-glucoside (**6**), 8-hydroxypinoresinol-4′-*O*-*β*-glucoside (**7**), and 8′-hydroxypinoresinol-4′-*O*-*β*-D-glucoside (**8**). Structural isomers **7** and **8** have been obtained as a mixture and were not separated. Compound **6** had previously been isolated from *Eucommia ulmoides*,[15] a traditional Chinese medicinal plant named "Tu-chung". Compounds **7** and **8** have been isolated from the same plant.[16] Compound **8** has also been obtained from *Olea europea* and other *Oleaceae*.[17,18]

All analytical data of previously described lignans **1**−**3** and **6**−**8** were in accordance with published data.

4′-*O*-*β*-D-Glucosyl-9-*O*-(6″-deoxysaccharosyl)olivil (4). Decoupled ^1H and ^{13}C NMR spectral data of compound **4** indicated a 7,9′-monoepoxylignan structure substituted with three sugar molecules (see Tables 1 and 2). This was confirmed by ^{13}C,^1H-coupled spectra and by ^1H,^1H-COSY

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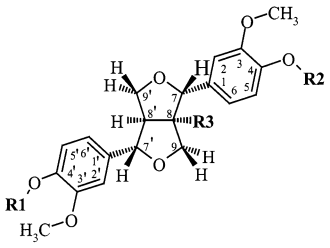
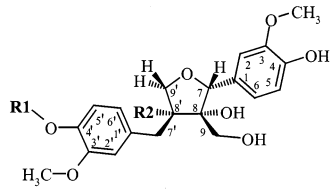
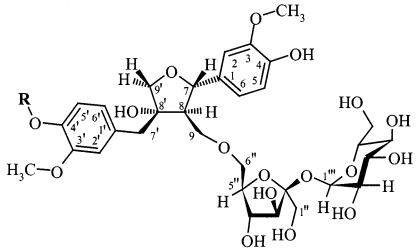


		R1	R2	R3
1	8-Hydroxypinoresinol	H	H	OH
2	Pinoresinol-4-O-β-D-glucoside	H	β-D-glucose	H
6	Pinoresinol-4,4'-di-O-β-D-glucoside	β-D-glucose	β-D-glucose	H
7	8-Hydroxypinoresinol-4'-O-β-D-glucoside	β-D-glucose	OH	OH
8	8-Hydroxypinoresinol-4-O-β-D-glucoside	H	β-D-glucose	OH



		R1	R2
3	Massoniresinol-4'-O-β-D-glucoside	β-D-glucose	OH
5	Berchemol-4'-O-β-D-glucoside	β-D-glucose	H



		R
4	4'-O-β-D-Glucosyl-9-O-(6"-deoxysaccharosyl)olivil	β-D-glucose

Figure 1. Structures of isolated lignans of *Valeriana officinalis* L.

spectra. The C9-methylene protons at 3.71 and 3.83 ppm were coupled to the proton at C8, which appeared as a multiplet at 2.30 ppm. C8 also coupled to the neighboring H7, which appeared as a doublet at 4.71 ppm. The methylene protons of C7′ and C9′ appeared as doublets. Thus, C8′ had to be substituted (hydroxylated), and it corresponded to the quarternary C atom at 82.4 ppm. An HMBC spectrum was recorded in order to clarify the attachment of the sugar molecules and to confirm the proposed basic structure. The quaternary C8′ coupled to the protons of the C7′ and the C9′ methylene groups, confirming the neighborhood of the atoms. Also, C9′ coupled with the protons at C7′. Furthermore, the axial proton at C9′ was in resonance with C7, completing the characterization of the tetrahydrofuran ring system. The aromatic 4-hydroxy-3-methoxyphenyl residues (characteristic fragment at 137 *m*/*z* in the EIMS spectrum) were connected to the tetrahydrofurane ring at C7 and C7′, as shown by coupling of the methylene protons at C7′ with the aromatic C1′ and C6′ signals on one hand and coupling of protons at C7 with C2 on the other hand. The assignment of the aromatic signals and the methoxy groups to one or the other aromatic system was done by HMBC and ^{1}H,^{1}H-COSY spectra (see Tables 1 and 2). Published spectral data for the natural product (−)-olivil (2*S*,3*R*,4*S*-configurated) are well in accordance with determined data for the core structure of compound **4**. According to Birch and Smith, the stereochemistry of olivil can be assigned via the shift for the 7-hydrogen;[19] it appeared at 4.72 ppm in case of an equatorial position of the aromatic substituent at C7 and an axial position of the hydroxymethyl group at C8. The axial position of both substituents resulted in a downfield shift for C7−H to 5.55 ppm. In compound **4** the C7−H appeared at 4.71 ppm, confirming the equatorial position of the aromatic substituent at C7 as in (−)-(2*S*,3*R*,4*S*)-olivil. While the chemical shifts for one of the phenylpropane partial structures of (−)-olivil and compound **4** were nearly identical, the signals for C1′, C3′, C4′, and C5′ of **4** were shifted downfield, indicating substitution of the oxygen atom at C4′. ^{13}C NMR data and their comparison with published data for glucose derivatives allowed the identification of a *β*-D-glucose attached at C4′. The anomeric proton H1′′′′ showed coupling to the aromatic proton H5′ in the NOESY experiment. Therefore due to the downfield shift of the C1′′′′ in **4** in comparison with unsubstituted *β*-D-glucose, the proposed attachment (C1′′′′−C4′−*O*-glucoside) was confirmed.[19] Olivil-4′-*O*-*β*-D-glucoside had previously been isolated from different plants, including *Ligustrum japonicum* (Oleaceae).[13,16,20] The ^{1}H NMR data indicated the presence of another glucose moiety, an α-D-glucose. The anomeric proton (H1′′′) at 5.38 ppm showed the typical shift and coupling (doublet, ^{3}J = 3.6 Hz) for α-D-glucose. This was confirmed by the ^{13}C,^{1}H-COSY spectrum with a signal at 93.6 ppm for the corresponding C1′′′ atom. Further characteristic signals indicated the presence of the disaccharide saccharose (α-D-glucopyranose and *β*-D-fructofuranose). Comparison with published NMR data for saccharose confirmed this assumption; however, the signal for C5′′ was shifted downfield in **4** as compared to *β*-D-fructofuranose due to its attachment to olivil. In NOESY experiments the expected coupling of the cis-configurated protons H1′′′ and H2′′′ of the α-D-glucose moiety was confirmed. Also, H3′′ of the *β*-D-fructofuranose residue was coupled with H5′′ and with the methylene protons at C1′′, H4′′, and C6′′. Interglycosidic coupling between the anomeric H1′′′ of the α-D-glucose residue and the H1′′ protons of the *β*-D-fructofuranose moiety confirmed the 1,2-*O*-glycosidic linkage. HMBC spectra also showed coupling between C2′′ and the anomeric H1′′′. The attachment of the saccharoside moiety to C9 of the olivil core was proven by NOE difference spectroscopy: Irradiation of proton H4′′ revealed its coupling to H9 (weak) and H6.

The aglycon (−)-(2*S*,3*R*,4*S*)-olivil has been isolated from several plants including the bark of *Eucommia ulmoides*[12] and from the bark and resin of *Olea europea*.[18,21] The fact that valerian also contains an olivil derivative is consonant with the chemotaxonomic relationship of the families of Valerianaceae and Oleaceae.

Berchemol-4′-*O*-*β*-D-glucoside (5). ^{1}H and ^{13}C NMR spectral data indicated a glucoside derivative of a 7,9′-monoexpoxylignan. The ^{13}C,^{1}H-COSY spectrum allowed the assignment of the C1′′ signal (103.1 ppm) and the attached proton (d, 4.86 ppm, J = 7.4 Hz) as the anomeric C atom of *β*-D-glucoside attached to the lignan core. The coupling pattern of the methylene protons at C9 (d, 3.60 ppm and d, 3.79 ppm) and the singlet at 4.82 ppm at C7 pointed to a C8−OH group corresponding to the signal for the quarternary C atom at 83.2 ppm. The coupling pattern in the ^{13}C,^{1}H-COSY spectrum revealed that C8′ was not hydroxylated. Aromatic NMR signals and the fragment ion of 137 *m*/*z* in the EI-mass spectrum indicated two 4-hydroxy-3-methoxyphenyl moieties. The ^{13}C NMR signal at 129.6 ppm was assigned to C1 since it was shifted upfield due to C8-hydroxylation. C1′ at 136.9 ppm was shifted

Table 1. ^{1}H NMR Spectral Data of Isolated Lignans[a]

proton	**3**[d]	**4**	**5**	**6**[e]	**7/8**[f]
2	7.03 d (J_m = 2.00 Hz)	7.13 d (J_m = 3.20 Hz)	6.92	7.02 d (J_m = 2.00 Hz)	6.72−7.16
5	6.71 d (J_o = 8.20 Hz)	6.73 d (J_o = 8.00 Hz)	6.73	7.14 d (J_o = 8.40 Hz)	6.72−7.16
6	6.75 dd (J_o = 8.10 Hz) (J_m = 2.00 Hz)	6.87 dd (J_o = 8.00 Hz) (J_m = 3.20 Hz)	6.73	6.91 dd (J_o = 8.50 Hz) (J_m = 2.00 Hz)	6.72−7.16
7	4.99 s	4.71 d (^3J = 7.30 Hz)	4.82 s	4.75 d (^3J = 4.20 Hz)	4.66 /4.72 s
8		2.30 m		3.11 m	
9	3.67 d 3.78 d (^2J = 11.5 Hz)	3.71 3.83	3.60 d 3.79 d (^2J = 11.4 Hz)	4.24 dd (J = 6.95 Hz) (J = 8.90 Hz)	
9eq					4.03/4.05 d (^2J = 4.90 Hz)
9ax					3.86[b]
2′	6.97 d (J_m = 1.90 Hz)	6.99 d (J_m = 3.20 Hz)	6.90 d (J_m = 2.00 Hz)	7.02 d (J_m = 2.00 Hz)	6.72−7.16
5′	7.09 d (J_o = 8.20 Hz)	7.08 d (J_o = 8.00 Hz)	7.09 d (J_o = 8.00 Hz)	7.14 d (J_o = 8.40 Hz)	6.72−7.16
6′	6.86 dd (J_o = 8.20 Hz) (J_m = 1.90 Hz)	6.84 dd (J_o = 8.00 Hz) (J_m = 3.20 Hz)	6.77 dd (J_o = 8.00 Hz) (J_m = 2.00 Hz)	6.91 dd (J_o = 8.50 Hz) (J_m = 2.00 Hz)	6.72−7.16
7′	2.94 d 3.01 d (^2J = 13.9 Hz)	2.95 d 3.03 d (^2J = 13.0 Hz)	2.54 dd 3.12 dd (^2J = 13.4 Hz) (^2J = 13.2 Hz)	4.75 d (^3J = 4.20 Hz)	4.77−4.83
8′			2.59 m	3.11 m	3.00−3.06 m
9′ax	3.65 d (^2J = 8.70 Hz)	3.59 d (^2J = 9.20 Hz)	3.63 dd (^2J = 8.50 Hz) (^3J = 5.50 Hz)	3.89[b]	3.74−3.78 dd (^2J = 9.10 Hz) (^3J = 6.40 Hz)
9′eq	3.83 d (^2J = 8.90 Hz)	3.80 d (^2J = 9.20 Hz)	4.05 dd (^2J = 8.50 Hz) (^3J = 6.50 Hz)	4.24 dd (J = 6.95 Hz) (J = 8.90 Hz)	4.43−4.47 dd (^2J = 9.00 Hz)
OCH_3	3.81 s	3.84 s	3.83 s	3.89 s	3.87/ 3.83 s
OCH_3′	3.86 s	3.87 s	3.86 s	3.89 s	3.84/ 3.88 s
1″	4.87 d (^2J = 7.50 Hz)		4.86 d (^2J = 7.40 Hz)	4.84 d (^3J = 7.20 Hz)	4.87/ 4.88 d (^3J = 7.20 Hz)
1‴		5.38 *d* (^3J = 3.2 Hz)		4.84 *d* (^3J = 7.20 Hz)	
1⁗		4.86[c]			

[a] Determined in CD_3OD at 500 MHz. [b] Beneath OCH_3 signal. [c] Beneath H_2O signal. [d] Compare ref 11. [e] Compound described in ref 15. No NMR data given. [f] Compare ref 16.

downfield, an indication that the glucose was attached to that phenyl ring. The HMBC spectrum showed methoxylation at C3′; thus glucosylation had to be at C4′. The ^{13}C,^{1}H-COSY spectrum allowed the assignment of the ^{1}H and ^{13}C signals (see Tables 1 and 2). NOE difference spectroscopy revealed that irradiation of the methoxy group (attached to C3) at 56.3 ppm gave an NOE effect only for the proton at 6.92 ppm (H2), proving the proposed substitution pattern of the phenyl ring. Furthermore, the ^{1}H,^{1}H-COSY spectrum showed no coupling partner for H2, while C3 exhibited coupling to the methoxy protons in the HMBC spectrum. The stereochemistry of compound **5** was assigned by the ^{1}H NMR data according to Birch and Smith.[19] The shift of H7 (4.82 ppm) was in accordance with the chemical shift for the analogously configurated dihydromelinol-II as described.

The valerian extract was found to contain 0.55% of lignans. The newly isolated lignans (compounds **3**−**8**) amounted to 0.35% of the extract (0.069% **3**, 0.053% **4**, 0.049% **5**, 0.109% **6**, 0.045% **7**, and 0.024% **8**), while the previously isolated lignans[10] **1** and **2** amounted to 0.20% as determined by quantitative HPLC.

Biological Activity. To obtain information about a potential contribution of the new lignans to the sedative effects of valerian, radioligand binding studies were performed at adenosine (A_1, A_{2A}), serotonin (5-HT_{1A}), benzodiazepine, and $GABA_A$ receptors. A_1 adenosine receptor affinity was initially determined in rat brain cortical membrane preparations using the A_1-selective radioligand [^{3}H]2-chloro-N^6-cyclopentyladenosine (CCPA); A_{2A} adenosine receptor affinity was measured in rat brain striatal membrane preparations with the A_{2A}-selective radioligand [^{3}H]3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine (MSX-2). Affinity to the benzodiazepine binding site of $GABA_A$ receptors was determined in rat brain cortical membranes versus [^{3}H]flunitrazepam, while affinity to the agonist binding site of the $GABA_A$ receptors was assessed in binding assays using [^{3}H]bicuculline methochloride ([^{3}H]BMC). Radioligand binding assays at serotonin 5-HT_{1A} receptors were performed with [^{3}H]8-hydroxy-2-(di-*n*-propylamino)tetralin ([^{3}H]8-OH-DPAT) at rat brain cortical membranes.

None of the isolated compounds significantly inhibited radioligand binding to 5-HT_{1A}, benzodiazepine, or $GABA_A$ receptors in concentrations up to 100 μM (data not shown). The mixture of the isomers **7** and **8** appeared to slightly enhance radioligand binding to 5-HT_{1A} and benzodiazepine receptors, while compound **6** (pinoresinol-4,4′-di-β-D-glucoside) appeared to enhance radioligand binding to $GABA_A$ receptors (23% at 10 μM) (data not shown). However, these enhancing effects were not concentration-dependent.

Inhibition of radioligand binding to A_1 and A_{2A} adenosine receptors was initially determined for lignans **3**, **4**, **5**, and a mixture of **7** and **8** in a single concentration (100 μM,

Table 2. ^{13}C NMR Spectral Data of Isolated Lignans[a]

C atom	**3**[b]	4	5	**6**[c]	**7**[d]	**8**[d]
1	128.7	134.9	129.6	137.5	127.3	131.7
2	112.8	111.5	112.7	111.7	112.7	113.5
3	149.7	149.1	148.7	151.0	149.5	150.5
4	146.7	147.5	149.1	147.5	147.7	150.0
5	116.5	115.8	116.0	118.1	116.4	117.7
6	121.9	120.7	121.6	119.8	121.7	121.3
7	86.4	85.7	85.7	87.1	89.4	88.9
8	82.0	61.9	83.2	55.5	92.7	92.9
9	64.5	60.7	64.5	72.8	76.3	76.0
OCH_3	56.3	56.3	56.3	56.8	56.3	56.7
1′	134.0	134.1	136.9	137.5	137.4	133.0
2′	116.4	116.1	114.5	111.7	112.0	111.3
3′	150.4	150.3	150.9	151.0	151.0	149.9
4′	146.7	146.6	146.4	147.5	147.6	149.5
5′	117.8	117.8	118.4	118.1	118.1	116.8
6′	124.2	123.9	122.3	119.8	120.2	120.7
7′	40.3	40.6	35.1	87.1	87.4	88.1
8′	82.3	82.4	51.7	55.5	62.4	62.3
9′	74.8	77.9	71.8	72.8	72.0	72.2
OCH_3′	56.8	56.7	56.8	56.8	56.7	56.3
1″	103.0	64.0	103.1	102.9	102.9	103.0
2″	75.0	105.3	75.0	74.9	74.9	74.9
3″	77.8	79.4	77.8	77.8	77.8	77.8
4″	71.4	75.7	71.4	71.3	71.4	71.4
5″	78.2	83.7	78.2	78.2	78.2	78.2
6″	62.5	63.3	62.5	62.5	62.5	62.5
1‴		93.6		102.9		
2‴		74.3		74.9		
3‴		74.6		77.8		
4‴		71.3		71.3		
5‴		73.2		78.2		
6‴		62.2		62.5		
1⁗		103.0				
2⁗		74.9				
3⁗		77.8				
4⁗		71.3				
5⁗		78.1				
6⁗		62.5				

[a] Determined in CD_3OD at 100 MHz, δ ppm. [b] Compare ref 11. [c] Compound described in ref 15; no NMR data given. [d] Compare ref 16.

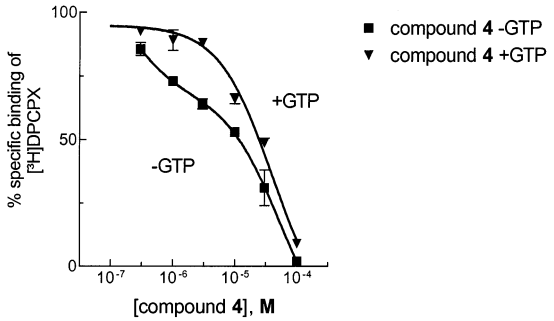


Figure 2. GTP shift experiment at human A_1 adenosine receptors expressed in Chinese hamster ovary (CHO) cells. Inhibition of binding of the antagonist radioligand [^{3}H]DPCPX by olivil derivative **4** was determined in the absence (■) and in the presence (▼) of 100 μM GTP. Without GTP two affinity states were detected with $K_H = 0.026 \pm 0.004$ μM (28 ± 13% of the receptors were detected in the high affinity state) and $K_L = 23.6 \pm 4.2$ μM. Fitted with a one-site binding model a K_i value of 10.1 ± 3.2 μM was calculated. GTP shifted the curve to the right and only one affinity state with a K_i value of 31 ± 3 μM was detected, which was comparable to K_L ($n = 2$).

see Table 3). Only 4′-*O*-β-D-glucosyl-9-*O*-(6″-deoxysaccharosyl)olivil (**4**) exhibited significant affinity for adenosine receptors and was more potent at A_1 as compared to A_{2A} adenosine receptors (Table 3). Therefore, olivil derivative **4** was further characterized with respect to its affinity and activity at A_1 ARs.

Adenosine is a neuromodulator in the central nervous system (CNS) acting via G-protein-coupled receptors, designated A_1, A_{2A}, A_{2B}, and A_3.[22] The main subtypes in the brain are A_1 and A_{2A}. Agonists at central A_1 and A_{2A} adenosine receptors (ARs) induce sedation, while antagonists, such as caffeine, exhibit central stimulatory effects.[22−24] The inhibitory neuromodulator adenosine is of considerable interest as a potential sleep factor because of pharmacological studies demonstrating sedative effects for adenosine agonists and powerful activating effects for caffeine and theophylline, known adenosine antagonists.[22−25]

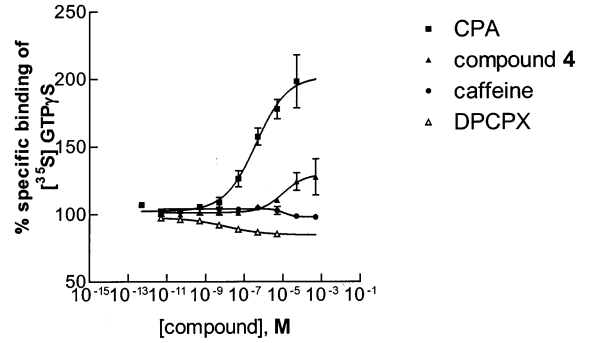


Figure 3. Adenosine A_1 receptor-mediated stimulation of [^{35}S]GTPγS binding to rat brain cortical membrane preparations induced by increasing concentrations of the olivil derivative **4** (▲, $EC_{50} = 3.98 \pm 0.42$ μM, maximal effect 126 ± 8%) compared to N^6-cyclopentyladenosine (CPA, ■, $EC_{50} = 0.017 \pm 0.002$ μM, maximal effect 193 ± 12%) as a full agonist, caffeine (●) as a neutral antagonist, and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, △, $IC_{50} = 0.006 \pm 0.0002$ μM, maximal effect, reduction to 86 ± 2% of GTPγS binding) as an antagonist with inverse agonistic activity.

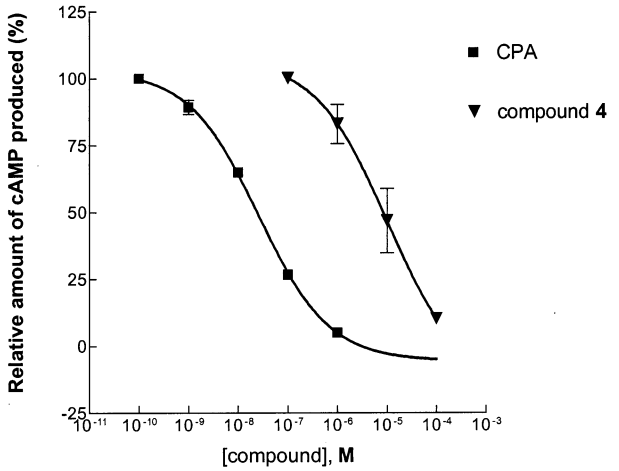


Figure 4. Inhibition of forskolin-stimulated cAMP accumulation by the A_1 adenosine receptor agonist N^6-cyclopentyladenosine (CPA ■, $IC_{50} = 0.024 \pm 0.002$ μM) and by olivil derivative **4** (▼, $IC_{50} = 15 \pm 5$ μM) determined in CHO-hA_1 membranes. Forskolin-stimulated cAMP was set at 100%; maximal inhibition was set at 0%. Inhibition of cAMP by compound **4** is given relative to the effect observed with CPA.

For olivil derivative **4**, a K_i value of 5.28 μM was determined at rat brain A_1 ARs using the agonist radioligand [^{3}H]CCPA (Table 3 and Figure 4). Thus, the A_1 affinity of **4** was severalfold higher than that of caffeine, a natural product that is believed to exert most of its pharmacological effects, such as CNS stimulation and diuresis, by a blockade of ARs. Compound **4** was additionally investigated in radioligand binding studies at human A_1 ARs stably expressed in Chinese hamster ovary (CHO) cells (Figure 2). In these experiments, an antagonist radioligand, [^{3}H]8-cyclopentyl-1,3-dipropylxanthine (DPCPX), was used, and competition curves were determined in the absence and in the presence of GTP (100 μM). Without GTP the curve for compound **4** was biphasic, showing K_i values of 26 nM for the high-affinity state and 23.6 μM for the

Table 3. Affinity of Isolated Lignans to A_1 and A_{2A} Adenosine Receptors

	A_1-receptor vs [^{3}H]CCPA rat brain cortical membrane		A_{2A}-receptor vs [^{3}H]MSX-2 rat brain striatal membranes
compound	percent inhibition of radioligand binding ± SEM at 100 μM (n = 2)	K_i ± SEM [μM]	percent inhibition of radioligand binding ± SEM at 100 μM (n = 3)
3	29 ± 6	n.d.[a]	0 ± 1
4	80 ± 5[b]	5.28 ± 0.68	49 ± 9
5	29 ± 6	n.d.	11 ± 6
7/8[c]	17 ± 5	n.d.	6 ± 5
caffeine	n.d.	23.5 ± 3.0[35]	58 ± 2 (K_i = 32.5 μM)[32]

[a] Not determined [b] n = 3. [c] Mixture of compounds **7**/**8** = ca. 3:2.

low-affinity state. GTP can cause an uncoupling of the receptor from the G-protein leading to a shift of the receptor from the high- to the low-affinity state for agonists.[26] The addition of GTP resulted in a rightward shift of the binding curve of **4**, which became monophasic. A K_i value of 31 μM was calculated in the presence of GTP, consistent with the K_i value for the low-affinity state. These results are a strong indication that olivil derivative **4** exhibits agonistic activity at human A_1 ARs.

[^{35}S]GTPγS binding assays can be used to assess antagonism, partial agonism, full agonism, and even inverse agonism of ligands of G-protein-coupled receptors.[27,28] While agonists stimulate the binding of GTPγS, a stable analogue of GTP, to the G-protein, neutral antagonists have no effect, and antagonists with inverse agonistic activity reduce GTPγS binding.[27]

Figure 3 shows the effects of the standard antagonists caffeine and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), and the agonist N^6-cyclopentyladenosine (CPA) on [^{35}S]-GTPγS binding to rat brain cortical membranes, which contain a high density of A_1 ARs. The agonist CPA clearly enhanced [^{35}S]GTPγS binding in a dose-dependent manner (EC_{50} = 16.9 ± 0.20 nM, maximal effect 193 ± 12%), while the antagonist caffeine had virtually no effect. DPCPX appeared to slightly decrease GTPγS binding by 14% to 86 ± 2% (IC_{50} = 6.03 ± 0.20 nM), indicating inverse agonistic activity. A similar effect had been shown in a cell line expressing the human A_1 AR in high density.[27]

Compound **4** increased [^{35}S]GTPγS binding (EC_{50} = 3.98 ± 0.42 μM); however, the maximal stimulation was lower (126 ± 8%) than that of the full agonist CPA. Thus, lignan **4** can be classified as a partial agonist at rat A_1 ARs.

The next step was to investigate compound **4** in a functional assay. A_1 ARs are coupled to adenylate cyclase (AC) in an inhibitory manner. We determined the inhibition of the forskolin-stimulated cAMP accumulation by the A_1 AR agonist CPA in comparison with the new natural product **4** (Figure 4). The experiments were performed using Chinese hamster ovary cell membranes containing the human A_1 AR. The A_1 agonist CPA dose-dependently inhibited cAMP accumulation with an IC_{50} value of 24 nM. Lignan derivative **4** showed the same effects with an IC_{50} value of 15 μM. Its maximal effect at the highest concentration tested appeared to be slightly lower than that of the full agonist CPA. In conclusion, lignan **4** clearly exhibits agonistic activity at human A_1 ARs.

All AR agonists, including partial agonists, that have been described so far are nucleosides or nucleoside analogues, mostly derivatives of adenosine.[29] The ribose moiety appears to be essential for high AR affinity and for agonistic activity. Since lignan **4** contains a fructofuranosyl residue, which bears resemblance to the ribofuranosyl residue of adenosine, it might be speculated that this part of the molecule is essential for activity at ARs. This hypothesis is supported by the fact that all other lignans that were tested do not exhibit this particular structure, and they did not show significant affinity for ARs. Massoniresinol-4′-*O*-β-D-glucoside (**3**), for example, a related lignan with one additional hydroxy group in position 8, but lacking the saccharosyl residue, was considerably less potent at A_1 and inactive at A_{2A} ARs in binding assays (Table 3).

In conclusion, we have isolated six new lignans of valerian, two of which are new natural products. One of the compounds, a novel olivil derivative, 4′-*O*-β-D-glucosyl-9-*O*-(6″-deoxysaccharosyl)olivil (**4**), was found to be a potent partial agonist at A_1 adenosine receptors. Compound **4** is the first example of a nonnucleoside adenosine receptor agonist, which is not structurally related to the physiological agonist adenosine.

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were obtained on a JEOL GX 400 D or a JEOL Eclipse 500 NMR spectrometer, respectively (Fa. JEOL, Eching, Germany). Compounds were dissolved in methanol-d_4. Chemical shifts are given in ppm referring to the δ-scale using tetramethylsilane, or the chemical shifts of the solvent, respectively, as internal standard. Decoupled and correlated (^{1}H,^{1}H-COSY, ^{1}H,^{13}C-COSY) spectra were recorded. HMBC (heteronuclear multiple bond correlation), NOESY (nuclear Overhauser enhancement spectroscopy), and NOE difference spectroscopy experiments were performed.

EI mass spectra were generated on a VG 7070H (Vacuum Generators) mass spectrometer supplemented with a Vector I (Teknivent) data system at 70 eV. For HRMS of compound **4** a FAB spectrum was obtained on a JEOL SX102 mass spectrometer (JEOL USA, Peabody, MA) operated at an accelerating voltage of 10 kV. Compound **4** was desorbed from a magic bullet matrix using 6 keV xenon atoms. Mass measurements in FAB were performed at 10 000 resolution using electric field scans with the sample peak bracketed by two cesium iodide reference ions. HRMS of compound **5** was done by electrospray ionization using a Micromass QTOF2 mass spectrometer (Micromass, Manchester, U.K.) after reductive amination using sodium cyanoborohydride. The mass spectrometer was calibrated with [Glu^1]-fibrinopeptide B human (Sigma-Aldrich, St. Loius, MO). Daunomycin (Sigma-Aldrich) was used as lock mass calibrant with an m/z = 528.1870. A resolution of 7500 was determined at m/z = 528. Mass Lynx 3.5 software was used for data processing.

Extraction and Isolation. Whole, dried *V. officinalis* roots of Polish origin (batch no. 39376) were obtained from M. Bauer Company, Vestenbergsreuth, Germany. Samples were deposited at the University of Marburg, Department of Pharmaceutical Biology. The finely ground roots of valerian (100 g) were divided into 10 g portions. Each portion was suspended in 100 mL of methanol and refluxed for 30 min. After cooling, the suspension was filtered, and the methanolic solution was evaporated under reduced pressure. The brown, oily residue (30% of original weight) was suspended in 50 mL of a mixture

of methanol and water (1:1, v/v). After centrifugation (10 min, 4000 rpm), the supernatant was decanted and evaporated. For preparative HPLC, the residue was dissolved in methanol/water (1:1, v/v) at a concentration of 0.5 g/mL. Preparative HPLC was performed on a Purospher RP 18, 10 μm column using a HPLC pump 64 system (Knauer, Berlin, Germany) with a 1.0 mL Rheodyne injection slope. Compounds were detected using a Merck Hitachi 655A variable-wavelength UV monitor. The flow rate was 15 mL/min; the wavelength of detection was λ = 280 nm, and a stepwise gradient was applied. Elution was performed with acetonitrile/water (17:83) for 9 min, followed by acetonitrile/H_2O (24:76) until 37 min. Fractions containing lignans were collected for further purification by preparative TLC on silica gel 60 F_{254} glass plates. The mobile phase consisted of $CHCl_3$/CH_3OH/toluene/NH_3 (10:6:3:1). The yields were as follows: 11.7 mg (compound **3**); 20.9 mg (compound **4**); 16.5 mg (compound **5**); 15.6 mg (compound **6**); 15.1 mg (compounds **7** and **8**). The following R_f values were determined: compound **3**, 0.17; compound **4**, 0.21; compound **5**, 0.25; compound **6**, 0.13; and compounds **7/8**, 0.30. The purity of the compounds was checked by analytical HPLC according to the method of Bodesheim[10] with modifications: The acetonitrile content of the gradient was reduced from 15% to 10% in order to detect the polar glycosylated lignans. Harpagoside was used as an internal standard.

4′-*O*-β-D-Glucosyl-9-*O*-(6″-deoxysaccharosyl)olivil (4): $[\alpha]_D^{21}$ −27° (*c* 0.05, DMSO); UV (MeOH/H_2O (1:1)) λ_{max} 227, 278 nm; for NMR spectral data, see Tables 1 and 2 and Results and Discussion; EIMS *m/z* 376 $[M]^+$ (olivil aglycon), 196, 152, 151, 137 (compare[30]); HRFABMS *m/z* 995.2112 (calcd for $C_{38}H_{54}O_{22}Cs$, 995.2161).

Berchemol-4′-*O*-β-D-glucoside (5): $[\alpha]_D^{21}$ −15° (*c* 0.01, DMSO); UV (MeOH/H_2O (1:1)) λ_{max} 225, 278 nm; IR (KBr) ν_{max} 3500, 2924, 2853, 1634, 1455, 1025 cm^{-1}; for NMR spectral data see Tables 1 and 2 and Results and Discussion; EIMS *m/z* 358 $[M]^+$ (aglycon − H_2O); HRESIMS *m/z* 561.2199 (calcd for $C_{26}H_{36}NNaO_{11}$, 561.2186) determined after reductive amination using $NaCNBH_3$.

Adenosine Receptor Binding Studies. Inhibition of [^{3}H]-CCPA (1.110 TBq/mmol, Amersham) binding to A_1ARs of rat cerebral cortical membranes and inhibition of [^{3}H]MSX-2 (3.145 TBq/mmol, Biotrend) binding to A_{2A} AR of rat striatal membranes was determined as previously described.[32,33] Briefly, frozen rat brain membranes were obtained from Pel-Freez (Rogers, Arkansas) and thawed at 4 °C. Cortex and striatum were dissected, and membrane fractions were prepared. Protein concentrations of ca. 70 μg/mL were used in the assays, as determined by the method of Bradford using a Biorad assay kit. Membranes were preincubated with 0.2 IU/mL of adenosine deaminase in order to remove endogenous adenosine. Nonspecific binding was determined in A_1 assays with 10 μM 2-chloroadenosine (amounting to less than 10%) in A_{2A} assays with 50 μM NECA (amounting to less than 20%). Incubations were carried out in a total volume of 1 mL of Tris-HCl buffer, pH 7.4 (50 mM), on a water shaking bath at 25 °C. In the A_1 assay incubation was terminated after 90 min, and in A_{2A} assays after 30 min by rapid filtration with a Brandell cell harvester through GF/B filters (Whatman). For A_{2A} assays filters were presoaked in 0.3% aqueous polyethylenimine solution for 30 min to reduce nonspecific binding. Filters were rinsed with 2 mL of ice-cold Tris-HCl buffer, pH 7.4 (50 mM). Radioactivity on the punched-out filters was measured in Ultima Gold (Canberra Packard) by liquid scintillation counting using a Tricarb 2100TR liquid scintillation analyzer (Canberra Packard). The Cheng Prusoff equation and K_i values were determined using Prism 3.00 (Graph Pad, San Diego, CA).

GTP Shift Experiments. Inhibition of binding of [^{3}H]-DPCPX (4.4 TBq/mmol, Amersham) by the lignan was measured in the presence and in the absence of 100 μM GTP.[33] Unlabeled DPCPX (10 μM) was used to determine nonspecific binding (amounting to less than 10%). The assays were carried out under the same conditions as described above for the A_1 assay, except for the use of membranes prepared from Chinese hamster ovary (CHO) cells stably transfected with the human A_1 AR (CHO-hA_1; protein concentration 20 μg/mL).

[^{35}S]GTPγS Binding Assay. Rat brain cortical membranes (7 μg per tube) or membranes of CHO-hA_1 cells (5 μg per tube), respectively, were incubated with 0.1−0.5 nM [^{35}S]GTPγS (46.3 TBq/mmol, NEN) in a total volume of 200 μL in 50 mM TRIS-HCl buffer, pH 7.4, containing 1 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM GDP, 100 mM NaCl, 2 IU/mL adenosine deaminase (ADA), 0.5% bovine serum albumin, and test drug according to Lorenzen et al.[28] Nonspecific binding was determined with 10 μM unlabeled GTPγS. As a control, 10 nM of adenosine was added to prove that the amount of ADA was sufficient to remove endogenous adenosine as well as the added adenosine (data not shown). Incubations were terminated after 45 min at 25 °C by the addition of 1 mL of ice-cold buffer containing 50 mM Tris-HCl, pH 7.4, and 5 mM $MgCl_2$ and rapid filtration through GF/B filters (Whatman) on a Brandel cell harvester, followed by two washing steps with cold buffer (2 mL each). Radioactivity on the filters was measured by liquid scintillation counting after transferring the punched out filters into 2 mL of scintillation cocktail (Ultima Gold).

Cyclic AMP Accumulation. Culture flasks with confluently grown cells were washed with PBS buffer, and cells were detached with a cell scraper. A membrane preparation of the cells was obtained according to the method described above. The P1 membrane fraction was resuspended in Tris-buffer 50 mM, pH 7.4, supplemented with 4 mM EDTA, 10 mM $MgCl_2$, and 5 μM GTP. The mixture was preincubated for 10 min at 37 °C with 2 IU adenosine deaminase to remove endogenous adenosine. Compounds dissolved in DMSO were diluted in 50 mM Tris-buffer, pH 7.4, containing EDTA (4 mM), $MgCl_2$ (10 mM), GTP (5 μM), forskolin (10 μM), and ATP (1 mM), and incubated for 10 min at 37 °C with 100 μL of the membrane suspension. Incubation was terminated by rapidly heating to 96 °C for 2 min. The tubes were cooled to room temperature and centrifuged at 2000*g*, 10 min, 4 °C, using an Allegra 21R centrifuge, Beckman Coulter, Germany. The supernatant (100 μL) was assayed in a charcoal adsorption assay using a commercially available kit (Amersham) to determine cAMP accumulation. Each experiment was carried out in duplicate up to three times.

Inhibition Experiments at 5-HT_{1A}, Benzodiazepine, and $GABA_A$ Receptors. Displacement assays were performed as previously described by Bodesheim et al.[10]

Cell Culture. CHO cells stably transfected with the human A_1 AR were grown adherently and maintained in Dulbecco's Modified Eagles Medium F12, adding 10% fetal calf serum, penicillin (100 IU/mL), streptomycin (100 μg/mL), and L-glutamate (2 mM) at 37 °C, 5% CO_2. Cells were grown to confluence and subcultured twice a week in a ratio of 1:5 or 1:20. For binding assays, culture medium was removed, and cells were washed with PBS and kept frozen at −80 °C until membrane preparation following the procedure described by Klotz et al.[34] For cAMP assays membranes were immediately used.

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