In Vitro Mammalian Arginase Inhibitory and Antioxidant Effects of Amide Derivatives Isolated from the Hempseed Cakes (*Cannabis sativa*)

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Abstract

In an effort to identify novel inhibitors of arginase, a phytochemical study was performed on hempseed cakes (*Cannabis sativa* L.). It led to the isolation of a new lignanamide, cannabisin I (**1**), together with seven known lignanamides, cannabisins A, B, C, F, M, 3,3′-demethylgrossamide, grossamide, and two phenylpropanoid amides, *N-trans*-caffeoyltyramine and *N-trans*-caffeoloctopamine, among which was later identified for the first time from *C. sativa*. Their structures were elucidated by comprehensive analysis of NMR spectroscopy and mass spectrometry data. These compounds were evaluated on mammal arginase (purified liver bovine arginase), showing that *N-trans*-caffeoyltyramine exhibited the higher activity with an IC_{50} value of 20.9 µM, which remains, however, less active than the reference compound *S*-(2-boronoethyl)-l-cysteine (IC_{50} = 4.3 µM). Radical scavenging capacity of these compounds was determined by the ORAC-FL method. All tested cannabisins displayed antioxidant activity close to or better than the reference compounds. *N-trans*-Caffeoyltyramine has both arginase inhibitory property and antioxidant capacity.

Key words

Cannabis sativa · Cannabaceae · hempseed · cannabisins · lignanamides · caffeoyl derivatives · arginase · antioxidant

Supporting information available online at http://www.thieme-connect.de/products

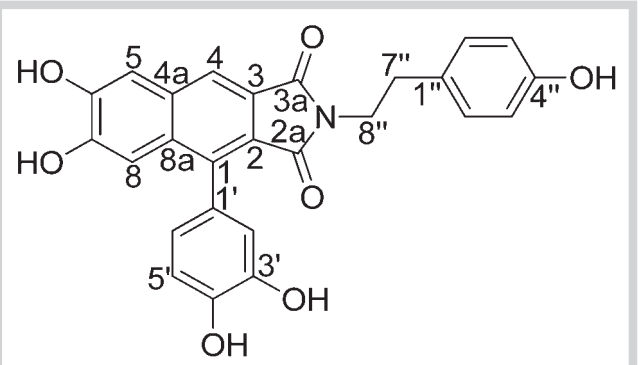

Fig. 1 Chemical structure of cannabisin I (**1**).

Arginase (amidinohydrolase, EC 3.5.3.1) is a metalloenzyme involved in the urea cycle by hydrolyzing L-arginine into urea and L-ornithine. By consuming L-arginine, arginase plays a crucial role in the biodisponibility of L-arginine to nitric oxide (NO) synthase, which also breaks down L-arginine into L-citrulline and NO, a key mediator of endothelial function [1]. Previous experimental studies showed that inhibition of arginase could raise NO bioavailability and reduce oxidative stress, and thus help to protect normal vascular function [2]. These beneficial effects have been largely demonstrated on animal disease models [3], but also via recent small-scale clinical studies [4–6] using local administration of arginase inhibitors and bringing the "proof of concept" for therapeutic use in human diseases. Among the few arginase inhibitors commercially available, two boronic acid derivatives [*S*-(2-boronoethyl)-L-cysteine (BEC) and 2-(*S*)-amino-6-boronohexanoic acid (ABH)] and one analogue of N-OH arginine [*N*ω-hydroxy-nor-L-arginine (nor-NOHA)] are the most potent. However, therapeutic use of these molecules has limitations mainly due to potential toxicity and a poor pharmacokinetic profile [7]. Therefore, the discovery of new structures of arginase inhibitors is needed. Plants constitute a valuable source for highlighting new active compounds in this field [8] and we previously showed that caffeic acid derivatives could be suitable as arginase inhibitor compounds [9]. Moreover, oxidative stress is also thought to play a crucial role in the onset and progression of endothelial dysfunction. Consequently, compounds able to simultaneously act as antioxidants and arginase inhibitors could be promising for the development of new drugs for the treatment of endothelial dysfunction involved in various diseases, including cardiovascular ailments. Industrial hemp (*Cannabis sativa* L.; Cannabaceae) is cultivated in many countries for textile and nutritional interest. Hempseed cakes, the by-products of this industry, are not currently considered as having high economic added value. But hempseed and its residue are sources of amide compounds derived from caffeic acid [10–13], among which some possess predominant radical scavenging activity [14]. With the aim to find novel natural compounds useful for the development of new treatments, we performed a biological and phytochemistry study of the extracts from hempseed cakes.

CH_2Cl_2 and MeOH crude extracts of hempseed cakes of *C. sativa* (7.5 kg) were evaluated on the arginase inhibition assay, and the MeOH extract exhibited the best activity (89% at 1 mg/mL and 56% at 0.1 mg/mL). Ten caffeoyl amide derivative compounds were isolated from the MeOH extract. Compound **1**, which we have named cannabisin I (**Fig. 1**), is new and was obtained as a yellow amorphous powder. The HRESIMS spectrum showed a quasi-molecular ion at *m*/*z* 456.10812 $[M - H]^-$ (calcd. for $C_{26}H_{18}NO_7$, 456.10812). From this data, the molecular formula $C_{26}H_{19}NO_7$ was deduced. The NMR spectra of **1** suggested a partial structure similar to that of cannabisin A, but with only one of the two tyramine moieties. In accordance with the molecular formula, the ^{13}C NMR data (**Table 1**) in combination with analysis of the HSQC spectrum revealed 26 carbon signals due to 2 methylenes, 10 aromatic methines, and 14 quaternary carbons (2 esters carbonyls, 5 oxygenated, and 7 olefinic). The ^1H, ^{13}C, and HSQC NMR spectra (**Table 1**) revealed signals attributable to arylnaphtalene and tyramine moieties. The tyramine moiety was supported by the HMBC correlations from H-2″ and H-6″ to C-1″ and C-4″ and from H_2-7″ to C-8″ and C-1″. Multiple HMBC correlations from H-4 to C-2a, C-2, C-1, C-8a, C-8, from H-5 to C-8a, C-4, and from H-8 to C-8a, C-1′ confirmed the presence of the arylnaphtalene moiety. The position of the substituents was deter-

Table 1 ^{1}H and ^{13}C NMR spectroscopic data for **1** (MeOD, 300/75 MHz, δ in ppm and J in Hertz).

Position	1*	
	δ ^{1}H	δ ^{13}C
1	–	122.4
2	–	133.1
2 a	–	169.4
3	–	126.7
3 a	–	169.4
4	7.88 *s*	122.8
4 a	–	128.3
5	7.23 *s*	113.5
6	–	150.9
7	–	150.9
8	7.04 *s*	112.3
8 a	–	133.1
1′	–	140.1
2′	6.66 *d* (*J* = 1,8 Hz)	118.4
3′	–	146.6
4′	–	146.2
5′	6.80 *d* (*J* = 8,0 Hz)	116.3
6′	6.53 *dd* (*J* = 8,0; 1,8 Hz)	116.4
1″	–	130.6
2″	6.89 *d* (*J* = 8,3 Hz)	131.0
3″	6.54 *d* (*J* = 8,3 Hz)	116.4
4″	–	157.2
5″	6.54 *d* (*J* = 8,3 Hz)	116.4
6″	6.89 *d* (*J* = 8,3 Hz)	131.0
7″	2.70 *t* (*J* = 7,4 Hz)	34.7
8″	3.64 *t* (*J* = 7,4 Hz)	40.6

mined by HMBC correlations (**Fig. 2**). The quaternary carbons at δ_C 150.9 were assigned at 6-OH and 7-OH on the basis of HMBC correlations from H-5 and H-8 to C-6, C-7. The positions of 3′-OH and 4′-OH were deduced from multiple HMBC correlations from H-2′, H-6′ to C-4′ and from H-5′ to C-3′. HRESIMS conjugated with NMR data suggested a five-membered imide ring to close the structure. Compound **1** was named cannabisin I and was isolated for the first time from a natural source. It was previously obtained by semisynthesis from cannabisin A by an acid treatment under strong conditions [Pyridine/HBr (48%), 90 °C during 2 days] [10]. The other purified compounds were identified as cannabisins A [10], B [11], C [11], F [12], M [14], 3,3′-demethylgrossamide [15], grossamide [10], *N-trans*-caffeoyltyramine [16], and *N-trans*-caffeoyloctopamine [17] by direct comparison of their spectral data with those in the literature.

Compound **1**, cannabisins B, F, and M, 3,3′-demethylgrossamide, and *N-trans*-caffeoyltyramine were first screened for their potential arginase inhibitory property at 10 and 100 µM on purified liver bovine arginase. It is noteworthy that, except for cannabisin F, all the evaluated compounds showed a more or less important inhibitory effect on our target (**Table 2**). From a structure-activity relationship point of view, we can notice that inactive cannabisin F is the only molecule that does not possess a catechol function. It confirms our previous finding that catechol is a crucial group for the arginase inhibitory activity [9]. Three compounds, *N-trans*-caffeoyltyramine, cannabisin B, and 3,3′-demethylgrossamide, showed inhibitory activity higher than 50% at 100 µM. Their IC_{50} values were thus calculated and gave, respectively, 20.9 µM, 126.8 µM, and 243.5 µM (**Table 2**). *N-trans*-Caffeoyltyramine is the most active compound, although it remains less active than BEC, which was used as a reference compound

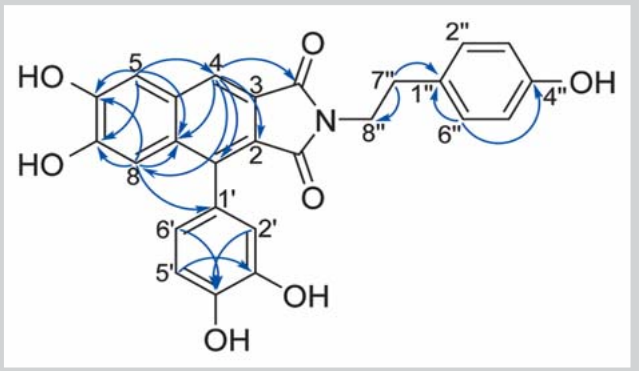

Fig. 2 Key HMBC correlations for **1**.

(4.3 µM). Recently, it was shown that lignanamides possess predominant radical scavenging activity [14,18]. Thus, we also evaluated these molecules for their antioxidant capacity through the well-known oxygen radical absorbance capacity (ORAC) method [19] using fluorescein as the fluorescent probe [20,21]. Except for cannabisins I and M, that are significantly less active than *trans*-ferulic acid and quercetin used as reference molecules, the tested compounds displayed antioxidant activity equivalent to that of quercetin (9.5 ± 0.8 Trolox equivalents) and better than that of *trans*-ferulic acid (6.4 ± 0.7 Trolox equivalents). *N-trans*-caffeoyltyramine (8.9 ± 0.6 Trolox equivalents), cannabisin B (9.8 ± 0.5 Trolox equivalents), and cannabisin F (8.9 ± 0.6 Trolox equivalents) gave higher activities (**Table 3**). The antioxidant activity arises from the caffeoyl and ferulic moiety. Interestingly, *N-trans*-caffeoyltyramine has both arginase inhibitory properties and antioxidant capacity.

In conclusion, the phytochemical study of hempseed cakes of *C. sativa* led to the isolation of eight lignanamides (cannabisins A, B, C, F, I, and M, grossamide, 3,3′-demethylgrossamide) and two phenylpropanoid amides, *N-trans*-caffeoyltyramine and *N-trans*-caffeoyloctopamine. These results showed that hempseed cakes constitute an interesting crude material for the extraction of biologically active compounds. Moreover, the combined properties of arginase inhibition and an antioxidant effect of *N-trans*-caffeoyltyramine make it a potentially interesting "lead compound" for the design and development of new drugs for the treatment of endothelial dysfunction associated with cardiovascular diseases.

Materials and Methods

Plant material

Hempseed cakes of *C. sativa* (Fedora 17 variety) were provided by La gOutte d'or du plateau, Le Fied, Franche-Comté, Jura (Batch number: T0020Ch). Hemps were cultivated under field conditions in Haute-Saône (EARL Jacquard, France), during the summer of 2014. The pressing of the seeds (performed at 20 °C) took place from October 27th to 29th.

Extraction and isolation

Hempseed cakes (7.5 kg) were deffated with petroleum ether (5 × 5 L) and successively extracted with methylene chloride CH_2Cl_2 (5 × 5 L), then methanol MeOH (5 × 5 L) at room temperature, leading to two extracts (49 g and 366 g, respectively). A part of the methanolic extract (287 g) was subjected to silica gel col-

Compounds	% Inhibition[a]		IC_{50} (µM)[b]
	C = 100 µM	C = 10 µM	
Cannabisin B	60.8 ± 2.6	21.5 ± 6.4	126.8 (102.9–156.2)
Cannabisin F	0.0 ± 6.4	5.4 ± 4.0	ND
Cannabisin I (1)	35.5 ± 4.1	8.4 ± 5.1	ND
Cannabisin M	26.0 ± 8.1	1.7 ± 9.1	ND
3,3′-Demethylgrossamide	50.9 ± 4.5	17.3 ± 5.8	243.5 (180.7–328.0)
N-trans-Caffeoyltyramine	62.5 ± 1.0	36.5 ± 5.7	20.9 (13.6–32.1)
BEC	ND	ND	4.3 (3.2–5.8)

ND: not determined. [a]All compounds were screened at 10 and 100 µM. Percentages of b-ARG 1 inhibition are presented as the mean ± SD (n = 3). [b]Results are presented as the mean of half-maximal inhibitory concentrations IC_{50} (95% confidence interval; n = 3)

Table 2 Arginase inhibition of cannabisins B, F, I, M, 3,3′-demethylgrossamide, and *N-trans*-caffeoyltyramine.

Table 3 Oxygen radical absorbance capacity (ORAC) of cannabisins B, F, I, M, and *N-trans*-caffeoyltyramine. Expressed as µmol of Trolox equivalents/µmol of pure compounds.

Compounds	Trolox equivalents
Cannabisin B	9.8 ± 0.5 [a***]
Cannabisin F	8.9 ± 0.6 [a***]
Cannabisin I (1)	4.3 ± 0.5 [a, b***]
Cannabisin M	5.0 ± 0.5 [a, b***]
N-trans-Caffeoyltyramine	8.9 ± 0.6 [a***]
Quercetin	9.5 ± 0.8
trans-Ferulic acid	6.4 ± 0.7

Results are expressed as the mean ± SD (n = 12). [a]Significantly different from *trans*-ferulic acid. [b]Significantly different from quercetin. ***P < 0.001

umn chromatography (CC) using a gradient of CH_2Cl_2-MeOH (10 : 0 to 7 : 3), leading to eight fractions (Frs. A–H). The repeated chromatographic purification of fraction F (39 g) resulted in the isolation of cannabisins A, B, C, F, I, M, 3,3′-demethylgrossamide, grossamide, *N-trans*-caffeoyltyramine, and *N-trans*-caffeoyloctopamine. The detailed purification procedure of these compounds is available in the Supporting Information.

Cannabisin I (**1**): Yellow amorphous powder; HR-ESIMS *m*/*z*: 456.10812 [M – H]$^-$ (calcd. for $C_{26}H_{18}NO_7$, 456.10812); ^{1}H-NMR (300 MHz, MeOD) and ^{13}C (75 MHz, MeOD), see **Table 1**.

Determination of arginase inhibition and IC_{50} values

We adapted the Corraliza et al. [22] method by miniaturizing the assay and by using purified bovine arginase. All the reagents were from Sigma-Aldrich except BEC, which was from Calbiochem (EMD Millipore), *trans*-ferulic acid, which was from TCI chemicals, and the purified liver bovine arginase 1, which was from MP Biomedicals. In each well of a microplate, the following were added: (1) 10 µL of a buffer containing Tris-HCl (50 mM, pH7.5) and 0.1% of bovine serum albumin (TBSA buffer), with or without (control) arginase at 0.025 U/µL, (2) 30 µL of Tris-HCl solution (50 mM, pH 7.5) containing $MnCl_2$ 10 mM, (3) 10 µL of a solution of the tested compound or its solvent (control), and (4) 20 µL of L-arginine (pH 9.7, 0.05 M). For the rapid preliminary screening test, two concentrations of the tested compounds (10 and 100 µM) were used. For IC_{50} determination, a range of ten concentrations was used from 10^{-7} to 5.10^{-3} M. The microplate was incubated for 60 min at 37 °C. The reaction was stopped by adding 120 µl of $H_2SO_4/H_3PO_4/H_2O$ (1 : 3 : 7). Thereafter, 10 µl of alpha-isonitrosopropiophenone (5% in absolute ethanol) were added and the microplate was heated at 100 °C for 45 min. After 5 min centrifugation then cooling, the absorbance was read at 550 nm using a spectrophotometer (Synergy HT BioTeck). For each tested compound, a stock solution (70 mM) was prepared in DMSO and then successively diluted in ultrapure water to get the desired concentrations. The values were obtained from three separate in duplicate (screening test) or in triplicate (IC_{50} values) experiments. The resulting absorbance was converted into the percentage of arginase inhibition, expressed as relative to the "100% arginase activity". For the determination of IC_{50} values, inhibition percentages were plotted on a semi-logarithmic scale and the IC_{50} values were estimated with Prism (v 5.0.3, GraphPad Software) by nonlinear sigmoidal curve fitting. Results of IC_{50} values are presented as the mean (95% confidence interval; n = 3).

Determination of antioxidant activity

The assay was performed in a 75-mM phosphate buffer at pH 7.4. The compound (20 µL; final concentration range of 0.1 to 1 µM) and fluorescein (120 µL; 70 nM final concentration) were incubated 15 min at 37 °C and (±)-6-hydroxy-2,5,7,8-Azobis(amidinopropane)dihydrochloride (AAPH) (60 µL; 12 mM final concentration) was rapidly added. Trolox (20 µL; final concentration range of 1 to 8 µM) was used as the standard. A blank, where the compound or trolox was replaced by phosphate buffer, was also carried out in each assay. The fluorescence was recorded every minute for 50 min on a microplate reader (Varioskan Flash Spectral Scanning Multimode Reader – Thermo electron) with 485 nm excitation and 520 nm emission. The area under the curve (AUC) was calculated as

$$AUC = 1 + \sum_{i=1}^{i=50} f_i/f_0$$

where f_i is the fluorescence reading at t = i min and f_0 is the fluorescence at t = 0 min. The net AUC was calculated by subtracting the AUC of the blank. Regression equations between net AUC and compound concentrations were determined by Microsoft Excel Program (Microsoft Office Standard 2010, Microsoft Corporation) and the relative ORAC values were calculated as the ratio of the standard curve of the compounds on the standard curve of Trolox. ORAC values are expressed as Trolox equivalents. All reaction mixtures were prepared in triplicate and at least four independent assays were performed for each compound. Values are presented as the means ± SD. Data were analyzed using GrapPad Prism version 5.03. Comparison between two values was assessed by unpaired Student's t-test. P < 0.05 was considered statistically significant.

Supporting information

General procedures, detailed extraction and isolation of the ten compounds as well as NMR data of **1** are available as Supporting Information.

Acknowledgements

We thank Michel Charreyron, oil producer, manager of "La goutte d'or du plateau" (Le Fied, Franche-Comté, Jura), for the supply of hempseed cakes. We are grateful to C. Vonthron-Sénecheau and J.-B. Gallé for the acquisition of the NMR data for *N-trans*-caffeoyloctopamine and Axel Marchal for the acquisition of the MS data for cannabisin M.

Conflict of Interest

The authors declare no conflict of interest.

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received April 12, 2016
revised October 5, 2016
accepted October 14, 2016

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DOI http://dx.doi.org/10.1055/s-0042-119400
Planta Med Int Open 2016; 3: e64–e67

ISSN 2199-157X

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