Structural Identification of a New Tri-*p*-coumaroylspermidine with Serotonin Transporter Inhibition from Safflower

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We previously reported that safflower (*Carthamus tinctorius* L.) ethyl acetate extract (HE) possessed an inhibitory action on serotonin (5HT) uptake in Chinese hamster ovary (CHO) cells expressing 5HT transporter (SERT) (S6 cells). Here, HE was adopted to go through an activity-guided isolation, and then an ingredient with potent SERT inhibitory action was obtained, which was elucidated as N^1,N^5-(*Z*)-N^{10}-(*E*)-tri-*p*-coumaroylspermidine (CX), a new coumaroylspermidine analog, by using spectroscopic methods including extensive 1D- and 2D-NMR analyses. Preliminary pharmacological study demonstrated that CX was a potent SERT inhibitor.

Key words *Carthamus tinctorius*; tri-*p*-coumaroylspermidine; transporter inhibitor

Safflower (*Carthamus tinctorius* L.), a member of the family Compositae, geologically distributes in all regions in China, and is utilized for producing herbal medicines for the treatment of inflammatory diseases, hyperlipemia, arteriosclerosis, gynecological disorders, and osteoporosis.[1)] To date, it has been known to contain flavonoids,[2)] cartharmin, safflower yellow A, quinochalone, safflomin, cartorimine, alkaloids,[3)] lignans, fatty acids,[4)] triterpene alcohols,[5)] and serotonin derivatives.[6—8)] Additionally, several coumaroylspermidine analogs also were found in safflower, *i.e.*, a principal analog N^1,N^5,N^{10}-(*E*)-tri-*p*-coumaroylspermidine (*EEE*) and its three *cis–trans* isomers [N^1,N^5,N^{10}-(*Z*)-tri-*p*-coumaroylspermidine (*ZZZ*), N^1(*E*)-N^5-(*Z*)-N^{10}-(*E*)-tri-*p*-coumaroylspermidine (*EZE*), and N^1(*E*)-N^5-(*Z*)-N^{10}-(*Z*)-tri-*p*-coumaroylspermidine (*EZZ*)],[9)] indicating that that some other geometric isomers of tri-*p*-coumaroylspermidine could exist in the natural plant. In our previous preliminary screening, we discovered that safflower extracts (especially ethyl acetate extract (HE) and *n*-butyl alcohol fraction) had regulatory actions on the monoamine transports in transporter-transgenic Chinese hamster ovary (CHO) cells, that is, activations of dopamine (DA)/norepinephrine (NE) uptake and an inhibition of serotonin (5HT) uptake,[10)] implying that there may be some constituent(s) acting as serotonin transporter (SERT) blocker(s). In this study, we thereby chose to isolate and characterize the structurally unknown, SERT-targeting compound(s) from the HE fraction by using an activity-directed extraction method and a series of NMR techniques. Finally, one of the active isolates was identified and elucidated as N^1,N^5-(*Z*)-N^{10}-(*E*)-tri-*p*-coumaroylspermidine (CX), a new tri-*p*-coumaroylspermidine as postulated previously by us.

Results and Discussion

The ethyl acetate extract of safflower (*Carthamus tinctorius* L.) was separated by column chromatography on silica gel and Sephadex LH-20 in combination with a concomitant bioactivity-tracing technique (detailed paradigm described in Supplemental) to afford a bioactive compound CX (designated as carthamide X by us) (Fig. 1). HPLC showed that the purity of CX sample was 95.6% (while the content of CX in HE was only 0.028%). Due to the sensitivity, validity, and feasibility,[10)] our screening platform previously established by using isotope-ligand-detection methods is suggested suitable for tracing some structurally unknown/unstable compounds with a sparse content in natural plants when combined with phytochemical techniques, which could, however, be hard to be isolated by traditional phytochemical method alone.

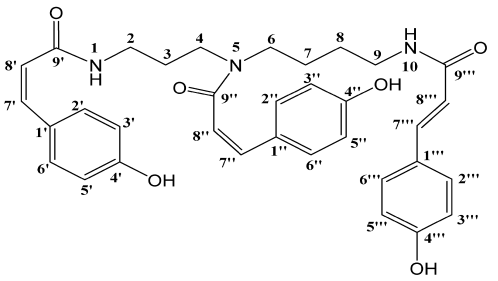

Fig. 1. Chemical Structure of N^1,N^5-(*Z*)-N^{10}-(*E*)-Tri-*p*-coumaroylspermidine

Numbers in the schematics, 1—10, 1′—9′, 1″—9″, and 1‴—9‴, referring to Table 1.

The SERT-targeting compound CX is white powders. Its IR spectrum showed absorption peaks for hydroxyl (3266 cm^{-1}), amide carbonyl (1650 cm^{-1}), and aromatic ring (1581, 1513 cm^{-1}). The molecular formula was determined as $C_{34}H_{38}N_3O_6$ by HR-electrospray ionization (ESI)-MS (*m*/*z* 584.2767, $[M+H]^+$, Calcd 584.2762). The ^{1}H-NMR spectrum (Table 1) exhibited signals for 6 olefinic, 12 aromatic, and 14 aliphatic protons together with two nitrogen protons at δ 8.05 and 7.95. The ^{13}C-NMR spectrum (Table 1) revealed signals for 3 amide carbonyl/6 olefinic carbons. Above information suggests compound CX is a coumaroylspermidine derivative.

Due to hindered rotation of the N^5-amidic bond, all ^{1}H-NMR spectra were rather complex. Thus, a variable temperature ^{1}H-NMR (at 95 °C) experiment was adopted, for the purpose of making signals easier to be distinguished. Analysis of ^{1}H–^{1}H correlation spectroscopy (COSY) spectrum deduced the presence of NH–$CH_2CH_2CH_2$ and NH–$CH_2CH_2CH_2CH_2$

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Table 1. ^{1}H- and ^{13}C-NMR Data for Compound CX (DMSO-d_6)

	δ_C	δ_H (J Hz)		δ_C	δ_H (J Hz)
1		8.05, 2H, m	9′	166.6	
2	37.0/38.8	3.12, 2H, m	1″	126.8	
3	26.2/27.7	1.70, 2H, m	2″,6″	130.4	7.22, 2H, d, 7.6
4	42.7/44.3	3.33, 2H, m	3″,5″	115.2	6.71, 2H, m
6	46.1/47.9	3.20, 2H, m	4″	158.0	
7	25.0/28.9	1.55, 1.59, 2H, m	7″	132.3	6.44, 1H, m
8	25.0/27.4	1.32, 1.41, 2H, m	8″	121.4	5.95, 1H, d, 12.5
9	36.5/38.5	3.02, 2H, m	9″	168.5	
10		7.95, m	1‴	126.4	
1′	126.8		2‴, 6‴	129.6	7.40, 2H, d, 7.2
2′, 6′	132.3	7.60, 2H, d, 7.2	3‴, 5‴	116.2	6.79, 2H, m
3′, 5′	115.7	6.71, 2H, m	4‴	159.2	
4′	158.3		7‴	139.0	7.32, 1H, d, 15.5
7′	136.6	6.49, 1H, m	8‴	119.1	6.37, 1H, m
8′	121.4	5.77, 1H, d, 12.9	9‴	165.8	

fragment, making assignment of H–N1 at δ 8.05, H-2 at 3.12, H-9 at 3.02, and H–N10 at δ 7.95, respectively. Despite overlapping in lower field, three *para*-substituted phenyl units were revealed by the presence of A_2X_2 type signals in ^{1}H- and ^{13}C-NMR spectra. Further study showed that six olefinic protons constructed three double bonds, two with *cis* forms (δ 5.77, d, J=12.9 Hz, H-8′; 5.95, d, J=12.5 Hz, H-8″) and one with *trans* form (δ 7.32, d, J=15.5 Hz, H-7‴). Several *p*-coumaroylspermidine derivatives have been reported,[9,11,12] among which the structural difference comes essentially from both the geometric isomerism of a C=C bond and the arrangement of three *p*-coumaroyl groups at N1, N5, and N10. To fix two *cis p*-coumaroyl groups and one *trans p*-coumaroyl group in the molecule, a heteronuclear multiple bond correlation (HMBC) experiment was carried out. The correlation of H-7′/C-9′, H–N1/C-9′, H-7″/C-9″, H_2-4/C-9″, H-7‴/C-9‴, and H–N10/C-9‴ were observed (Fig. 2), determining compound CX as N^1,N^5-(*Z*)-N^{10}-(*E*)-tri-*p*-coumaroylspermidine (Fig. 1), a new *p*-coumaroylspermidine.

Although safflower has been reported to contain flavonoids,[2] cartharmin, safflower yellow A, quinochalone, safflomin, cartorimine, alkaloids,[3] lignans, and fatty acids,[4] there are few reports showing that safflower contains coumaroylspermidine analogs, a family of hydroxycinnamic acid amides. The principal tri-*p*-coumaroylspermidine, *i.e.* N^1,N^5,N^{10}-(*E*)-tri-*p*-coumaroylspermidine, was first identified from flowers of *Cratuegus* in 1978[13]; afterwards, this *EEE* isomer was isolated successively from *Rosaceae*,[14] *Quercus dentata* pollen,[15] anthers of *Aphelandra tetraffona* and *A. chamissoniana* (a *ZZZ* isomer also was isolated concomitantly),[12] sunflower (*Helianthus annuus* L.) pollen,[16] and Brazilian bee pollen.[17] Pharmacologically, this principal compound has been reported to have anti-human immunodeficiency virus (HIV) infection,[11,18] anti-*Helicobacter pylori*,[19] anti-tumor,[20,21] anti-fungal,[22] and antioxidant activities.[17] More recently (by the year of 2008), several tri-*p*-coumaroylspermidines with property of *cis–trans* conformation such as the *ZZZ*, *EEE*, *EZE*, and *EZZ* isomer have also been found in safflower [the florets of *Carthamus tinctorius* (Compositae)].[9] However, until now, the other related geometric isomers of the principal compound have not yet been reported in natural plants especially safflower. In our current study, the coumaroylspermidine isomer CX with *ZZE* configuration was first isolated and identified from safflower (*Carthamus tinctorius* L.). Primary pharmacological study demonstrated that this first-reported compound CX had a selective antagonistic activity on SERT, on the basis of the fact that it acted toward 5HT but not NE, DA, or γ-aminobutyric acid transport (data not shown) in respective transporter-transformed CHO cells. Since SERT is thought to be involved in the pathogenesis of several affective disorders,[23,24] we suggest that CX, as a potent 5HT-uptake blocker (IC_{50}=0.54±0.15 μM), could possess antidepressant action and would be helpful in improving neuropsychological disorders.

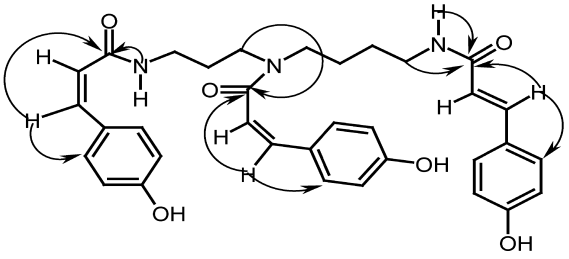

Fig. 2. Key HMBC Correlation of Compound CX

Collectively, safflower contains the SERT-targeting isolate that is N^1,N^5-(*Z*)-N^{10}-(*E*)-tri-*p*-coumaroylspermidine. The cinnamonyl chromophores for the mono- or di-*p*-coumaroylspermidines have been reported to undergo (*E*)→(*Z*) isomerization,[25] suggesting that there still may be three additional tri-*p*-coumaroylspermidine geometric isomers (*ZEZ*-, *ZEE*-, and *EEZ*-isomer) naturally occurring in plants especially safflower besides our currently reported *ZZE* isomer and the previous reported *ZZZ*, *EEE*, *EZE*, and *EZZ* isomer.[9] This hypothesis is supported likely by the fact that there still are several SERT-targeting, non-purified fractions shown in the process of either column chromatographic fractionation of HE (data not shown) or previous organic solvent extraction of safflower material.[10] For further study, these putative *cis–trans* isomers will be isolated from safflower by adoption of a more sensitive extraction technique; and then their detailed pharmacological profiles will be established, by which a quantitative structure–activity relationship could be deduced and a subsequent structural modification of lead isomer to a robust compound would be directed in near future.

Experimental

General The IR spectrum was recorded on an IMPACT 400 (KBr) spectrometer (Nicolet, U.S.A.). The ^{1}H- and ^{13}C-NMR spectra were run on Varian INOVA-500 spectrometers (^{1}H, 500 MHz, ^{13}C, 125 MHz) (Varian, Palo Alto, CA, U.S.A.) with TMS as an internal standard, and the HR-MS was on Finnigan LTQ FT mass spectrometer (Thermo Electron). The purity of CX was analyzed by a normalization method following HPLC (1200 series, Agilent technologies, Santa Clara, CA, U.S.A.) detection using ZORBAX SB-C18 reversed-phase column (4.6 mm×250 mm, 5 μm, i.d.) and detector at 254 nm, under conditions of methanol/H_2O=54/46 (v/v) as mobile phase and 1.0 ml/min as flow rate. HR-ESI-MS spectrum was determined on Mariner mass spectrometer (PerSeptive Biosystems, Framingham, MA, U.S.A.). Silica gel (200—300 mesh) (used for column chromatography) and pre-coated silica GF254 plates (used for TLC) were purchased from Qingdao Marine Chemical Co., Ltd. Sephadex LH-20 was from Pharmacia, Sweden. All solvents were in chemical or analytical grades (Shanghai Chemical Co., Ltd.). Dry safflower material (voucher no. 20050218) was commercially provided from Henan Province, China, deposited at the Herbarium of Cell-Star Bio-Technologic Co., Ltd. (Shanghai) and identified by an expert herbalist at the Institute of Materia Medica, Shanghai Institutes for Biological Sciences.

Isolation of Free CX Isolation of active compound(s) from safflower (*Carthamus tinctorius* L.) was seen.

Structure Determination Compound CX is white powders. $[\alpha]_D^{20} \pm 0.0°$ (c=0.040, MeOH), IR ν_{max} cm^{-1} (KBr): 3266 (hydroxyl), 1650 (amide carbonyl), 1581 and 1513 (aromatic ring); HR-ESI-MS *m/z*: 584.2767, $[M+H]^+$ (Calcd for $C_{34}H_{39}N_3O_6$, 584.2762). ^{1}H- and ^{13}C-NMR spectra (see Table 1).

[^{3}H]5HT Uptake Assay *in Vitro* 5HT-uptake assay on CHO cells expressing the rat SERT (rSERT) (S6 cells) was used in this study as described previously.[26—28)]

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