Tetra-*cis*/*trans*-Coumaroyl Polyamines as NK_1 Receptor Antagonists from *Matricaria chamomilla*

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Supporting information 1D and 2D NMR spectra of **1a**/**1b**, **2a**/**2b** and **3a**/**3b** are available online at http://www.thieme-connect.de/products.

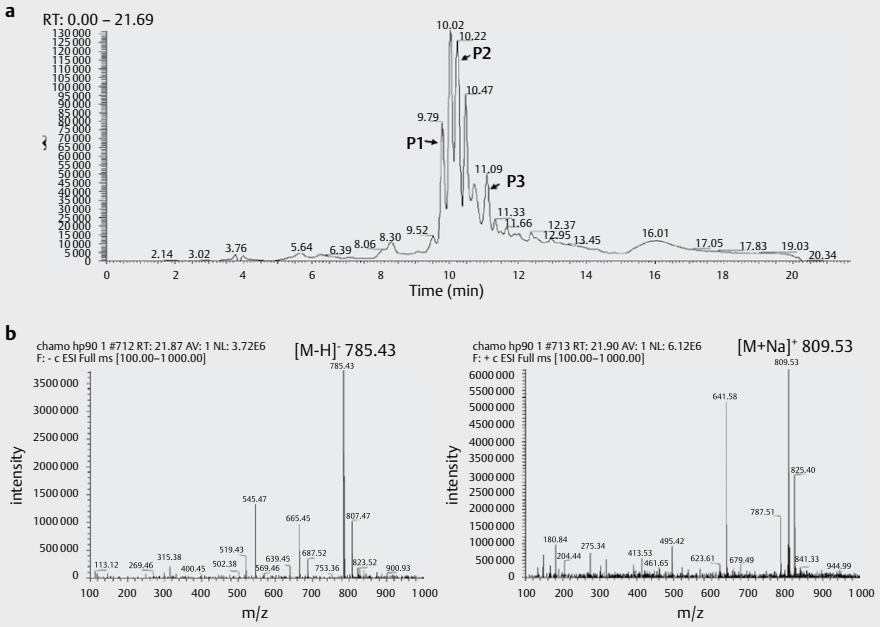
ABSTRACT

Cis-trans isomers of N^1,N^5,N^9,N^{14}-tetra-*p*-coumaroyl thermospermines and N^1,N^5,N^{10},N^{14}-tetra-*p*-coumaroyl spermines were found in *Matricaria chamomilla*, German chamomile using countercurrent chromatography with methylene chloride-methanol-water (1:1:1, v/v/v). Their structures were elucidated based on spectroscopic and spectrometric data (1D and 2D NMR, and HRESIMS). The antagonistic activity against the neurokinin-1 receptor was examined by a calcium assay, measuring the cytosolic fluorescence triggered by substance P binding to the receptor. The compounds **1a**/**1b**, **2a**/**2b**, and **3a**/**3b** potently suppressed the calcium flux compared to the known antagonist L-703,606 oxalate, indicating that the compounds competitively inhibited the binding of substance P. They also suppressed substance P-induced proliferation in MDA-MB-453, the HER2-amplified breast cancer cell line. It is suggested that tetracoumaroyl thermospermines and tetracoumaroyl spermines are promising antagonists, exerting positive effects on substance P/neurokinin-1 receptor-related diseases.

Introduction

Chamomile belongs to the Asteraceae family, and the species most commonly used as tea is *Matricaria chamomilla* L., known as German chamomile. It has been traditionally consumed to treat sleep disorders and to ameliorate anxiety and depression [1]. A broad range of biological activities attributed to chamomile has been studied in modern research, which has demonstrated that chamomile possesses antiallergic, anticancer, anti-inflammatory, and wound healing properties [2]. More than 120 chemical compounds involved in the bioactivities of chamomile have been identified, including flavonoids, terpenoids, and coumarins [3]. Tetracoumaroyl spermine (N^1,N^5,N^{10},N^{14}-tetrakis[3-(4-hydroxyphenyl)-2-propenoyl]-1,5,10,14-tetraazatetradecane) was first isolated from Asteraceae plants by preparative HPLC, and its potency as a neurokinin-1 (NK_1) receptor antagonist was demonstrated by the inhibition of substance P-induced contractions in the guinea pig ileum [4]. The *cis-trans* isomers of tricoumaroyl spermidine were separated from safflower using high-speed countercurrent chromatography (HSCCC) [5], and the antioxidant activities of tetracoumaroyl spermine (TCS) and tricoumaroyl spermidine have also been reported [6]. Thermospermine is the isomer of spermine converted from spermidine by thermospermine synthase [7], and it is abundant in the plant kingdom [8, 9]. Despite occurring in the majority of plants, hydroxycinnamic acid-conjugated thermospermine has not yet been reported. The antagonism of tetracoumaroyl thermospermine (TCTS) is deducible by the structural analogy with TCS; hence, the isolation and characterization of novel TCSs/TCTSs would be worthwhile for NK_1 receptor-targeted drug development.

The NK_1 receptor is one of the G protein-coupled receptors (GPCR), and the activation or deactivation of the receptor through the binding of an agonist/antagonist can be quantitatively assessed by intracellular calcium tagged with fluorescence. The activated GPCR triggers phospholipase C to hydrolyze the membrane phospholipid PIP_2 to form IP_3, and the IP_3 binds to its receptor, releasing calcium from the endoplasmic reticulum [10]. The cytosolic calcium level is an indicator for the transient changes induced by the binding of substance P to the NK_1 receptor. NK_1 receptors and substance P are found in the brain regions that regulate emotion, such as the hypothalamus, amygdala, and the periaqueductal gray [11].



▶ **Fig. 1** HPLC chromatogram and mass spectra of the tetra-*cis/trans*-coumaroyl polyamines. 3 peaks possessing isomers of hydroxycinnamates-conjugated polyamines were detected by LC-ESI/MS **a** and the compounds exhibited the identical molecular weight, where the *m/z* values at $[M-H]^-$ 785 and $[M+Na]^+$ 809 estimated the formula as $C_{46}H_{50}N_4O_8$ **b**.

The binding of substance P to NK_1 receptors is also related to the transmission of stress signals, inducing mood disorders and anxiety [12, 13]. NK_1 receptor antagonists selectively suppress the substance P-mediated actions, showing antidepressant, anxiolytic, and antiemetic properties [14]. The NK_1 receptor/substance P complex is also widely distributed in tumor cells, and the binding stimulates mitogenesis, inducing proliferation and inhibiting apoptosis through the mitogen-activated protein kinase (MAPK) pathway. The NK_1 receptor antagonists L-732,138, L-703,033, and aprepitant have shown antitumor activity in human cancer cell lines [15, 16]. In breast cancer, substance P and NK_1 receptors are involved in the acquisition of oncogenicity [17, 18], and substance P enhances the aggressiveness of breast cancer cells by promoting the activity of the receptor tyrosine-protein kinase ERBB family, including epidermal growth factor receptor 1 (EGFR) and 2 (HER2) [19]. It was also found that substance P and NK_1 receptors are highly expressed in $HER2^+$ primary breast tumors [20]. Breast cancer can be classified based on the immunohistochemical expression of estrogen receptors (ER), progesterone receptors (PR), and HER2 [21]. In this study, 2 subtypes of breast cancer cell lines (HER2-positive MDA-MB-453 and MDA-MB-231, characterized by the lack of expression of ER, PR, and HER2) were used to demonstrate that the antagonism against NK_1 receptors inhibits substance P-induced proliferation in HER2-positive breast cancer.

Results and Discussion

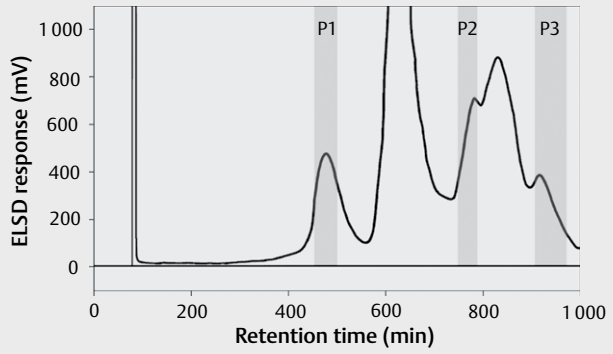
3 peaks that represented identical ions were detected on liquid chromatography-electrospray ionization mass spectrometry (LC-ESI/MS), and the *m/z* values were estimated at $[M-H]^-$ 785.43 and $[M+Na]^+$ 809.53 (▶ **Fig. 1**). Peaks 1, 2, and 3 (P1, P2, and P3) were revealed to be the isomers of TCS by comparing them to the molecular weights of reported compounds from chamomile [4]. Countercurrent chromatography (CCC) was used to separate the isomers due to the highly similar polarities among the compounds, which impede the separation by reversed-phase C_{18} chromatography. The partition coefficients of P1, P2, and P3 were first examined with chloroform-methanol-water (1:1:1, v/v/v) [5] that was used to separate the *cis-trans* isomers of tricoumaroyl spermidine. The solvent composition was modified (▶ **Table 1**), and methylene chloride-methanol-water (1:1:1, v/v/v) was finally selected. P1, P2, and P3 were collected at 440, 750, and 920 min of the retention time on the CCC chromatogram (▶ **Fig. 2**). The isomeric compounds presented unique UV spectra (▶ **Fig. 3**), showing that the UV λ_{max} values moved to longer wavelengths, implying that their structural differences were derived from *cis-trans* hydroxycinnamic acid groups conjugated on the tetra-amines. Meanwhile, P1, P2, and P3 were found to possess the unique mass spectral fragment exhibiting the $[M-H]^-$ ion at *m/z* 144.4. P1, P2, and P3 were originally regarded as the isomers of spermine-based tetracoumaroyl moiety-conjugated compounds; however, the ions were estimated to be relationally generated from thermospermine due to the asymmetric carbon arrangement (▶ **Fig. 4**).

The integration in the ^1H NMR data of P1, P2, and P3 showed the double numbers of hydrogen atoms, 8 olefins and 20 non-overlapping aliphatic protons, suggesting the co-presence of TCS and TCTS in P1, P2, and P3 (▶ **Table 2**, **3**). The ^1H and ^{13}C NMR spectra were compared with previous data [4, 6]. In the ^1H NMR spectrum of P1 (**Fig. S1**, Supporting Information), 16 protons, at δ_H 6.49 (d, J = 12.7 Hz), 6.48 (d, J = 12.8 Hz), 6.45 (d, J = 12.8 Hz), 6.38 (d, J = 12.7 Hz), 5.95 (d, J = 12.8 Hz), 5.83 (d, J = 12.8 Hz), 5.79

▶ **Table 1** Partition coefficients of P1, P2, and P3.

MC	Chl	EA	IPA	ACN	EtOH	MeOH	DW	P1[a]	P2[a]	P3[a]
1.250						1.000	1.000	1.206	2.065	2.666
1.125						1.000	1.000	1.465	2.173	2.685
1.000						1.000	1.000	1.199	2.293	2.782
1.000			0.250			0.750	1.000	0.617	0.795	0.984
1.000				0.250		0.750	1.000	0.387	0.361	0.353
1.000					0.250	0.750	1.000	0.214	0.165	0.174
0.875	0.125					1.000	1.000	1.034	2.239	1.881
0.500	0.500					1.000	1.000	0.608	1.382	1.099
0.125	0.875					1.000	1.000	0.653	0.802	0.933
	1.000					1.000	1.000	0.546	0.925	0.964
	0.875	0.125				1.000	1.000	0.479	0.484	0.481

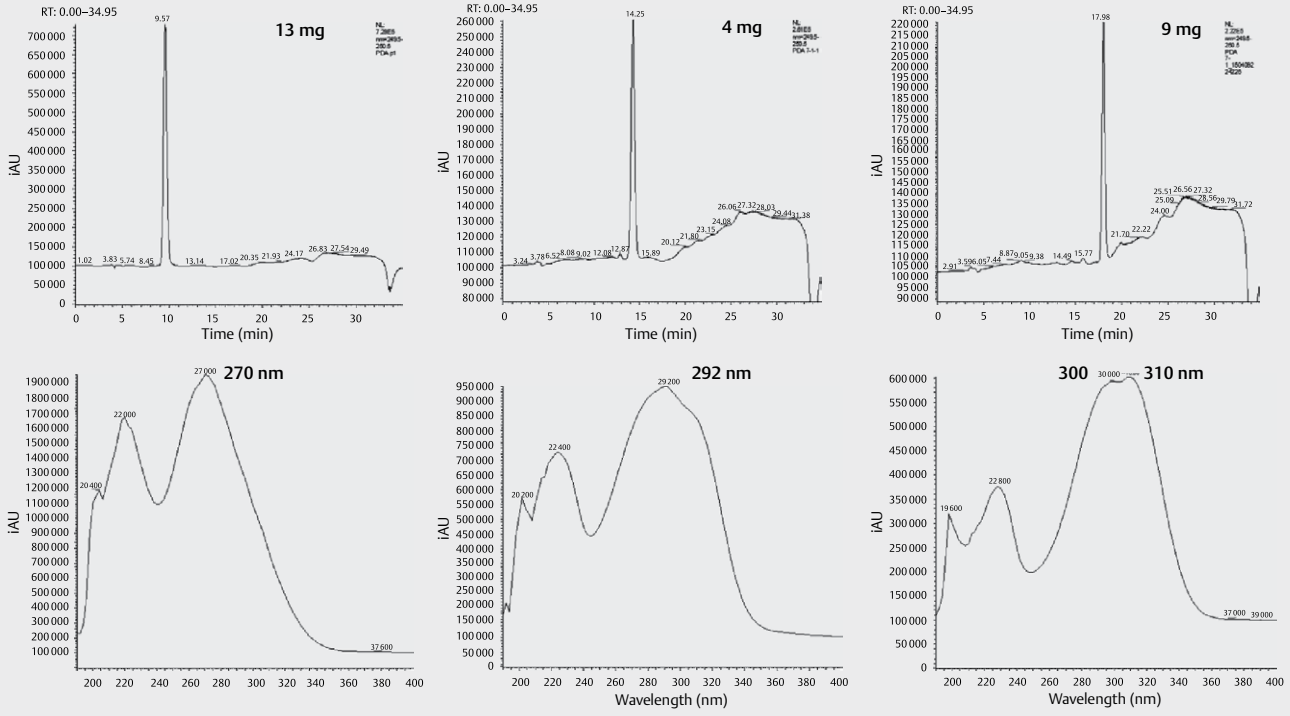
[a] Partition coefficients (K_D values) of P1-3 were evaluated using HPLC by the area integration of the upper phase divided by that of the lower phase on the chromatogram



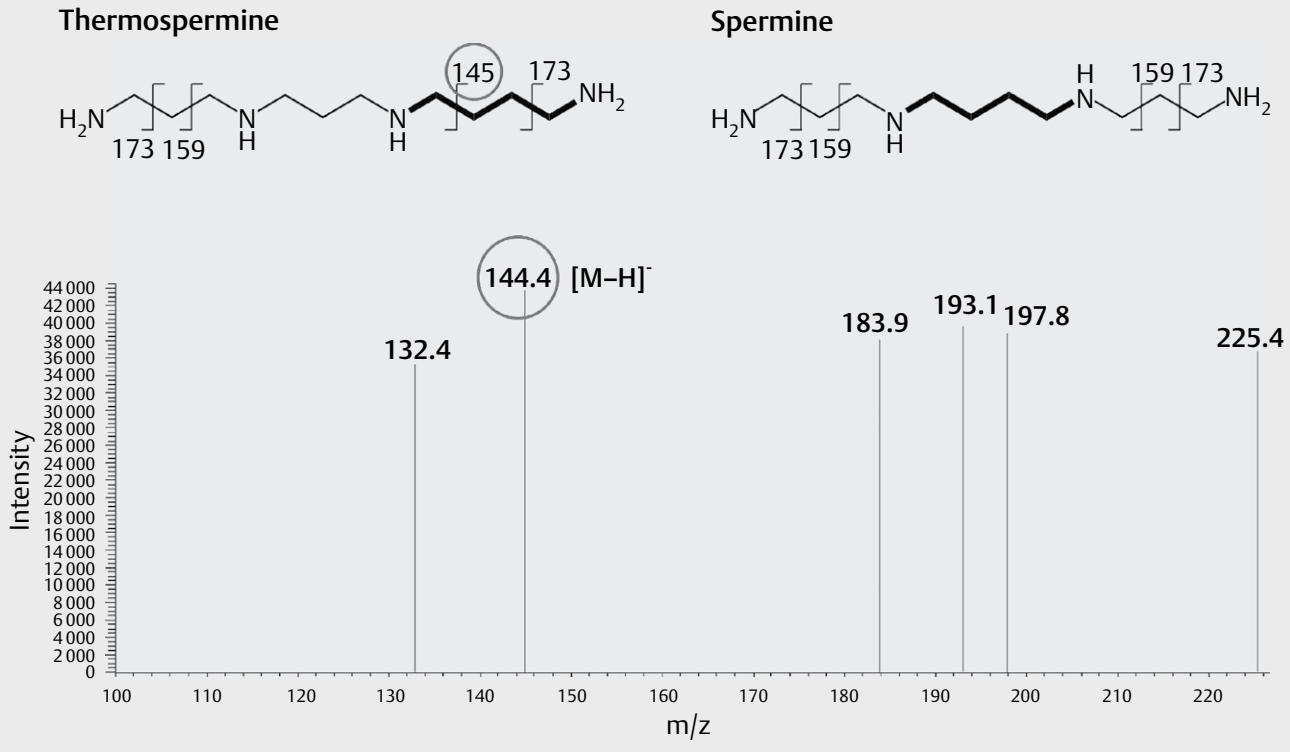
▶ **Fig. 2** CCC chromatogram of the tetra-*cis*/*trans*-coumaroyl polyamines. Methylene chloride/MeOH/water (1:1:1, v/v/v) was selected for the CCC operation to separate hydroxycinnamates-conjugated polyamines.

(d, J = 12.8 Hz), 5.75 (d, J = 12.8 Hz), 6.51 (d, J = 12.7 Hz), 6.50 (d, J = 12.8 Hz), 6.40 (d, J = 12.8 Hz), 6.38 (d, J = 12.7 Hz), 5.92 (d, J = 12.8 Hz), 5.87 (d, J = 12.8 Hz), 5.78 (d, J = 12.8 Hz), and 5.74 (d, J = 12.8 Hz), indicated 8 *cis*-coumaroyl moieties. Based on the assignments with the heteronuclear single-quantum correlation (HSQC) and heteronuclear multiple-bond correlation (HMBC) experiments, the 2 polyamines in P1 were identified to be $N^1(Z)$-$N^5(Z)$-$N^{10}(Z)$-$N^{14}(Z)$-tetra-*p*-coumaroyl spermine and $N^1(Z)$-$N^5(Z)$-$N^9(Z)$-$N^{14}(Z)$-tetra-*p*-coumaroyl thermospermine, which were named **1a** and **1b**, respectively. In the ^{1}H NMR spectrum of P2 (**Fig. S7**, Supporting Information), 16 protons, at δ_H 7.33 (2H, d, J = 15.7 Hz), 6.45 (d, J = 12.6 Hz), 6.41 (d, J = 15.7 Hz), 6.38 (d, J = 12.6 Hz), 6.40 (d, J = 15.7 Hz), 5.95 (d, J = 12.6 Hz), 5.84 (d, J = 12.6 Hz), 7.30 (2H, d, J = 15.7 Hz), 6.41 (d, J = 12.6 Hz), 6.39 (d, J = 12.6 Hz), 6.36 (2H, d, J = 15.7 Hz), 5.93 (d, J = 12.6 Hz), and 5.89 (d, J = 12.6 Hz), indicated 4 *cis*- and 4 *trans*-coumaroyl moieties. Based on the assignments with the HSQC and HMBC experiments, the 2 polyamines in P2 were identified to be $N^1(E)$-$N^5(Z)$-$N^{10}(Z)$-$N^{14}(E)$-tetra-*p*-coumaroyl spermine and $N^1(E)$-$N^5(Z)$-$N^9(Z)$-$N^{14}(E)$-tetra-*p*-coumaroyl thermospermine, which were named **2a** and **2b**, respectively. In the ^{1}H NMR spectrum of P3 (**Fig. S13**, Supporting Information), 16 protons, at δ_H 7.41 (2H, d, J = 15.7 Hz), 7.34 (2H, d, J = 15.7 Hz), 6.92 (d, J = 15.7 Hz), 6.90 (d, J = 15.7 Hz), 6.42 (d, J = 15.7 Hz), 6.41 (d, J = 15.7 Hz), 7.40 (2H, d, J = 15.7 Hz), 7.31 (2H, d, J = 15.7 Hz), 6.83 (d, J = 15.7 Hz), 6.82 (d, J = 15.7 Hz), and 6.40 (2H, d, J = 15.7 Hz), indicated 8 *trans*-coumaroyl moieties. Based on the assignments with the HSQC and HMBC experiments, the 2 polyamines in P3 were identified to be $N^1(E)$-$N^5(E)$-$N^{10}(E)$-$N^{14}(E)$-tetra-*p*-coumaroyl spermine and $N^1(E)$-$N^5(E)$-$N^9(E)$-$N^{14}(E)$-tetra-*p*-coumaroyl thermospermine, which were named **3a** and **3b**, respectively. All chemical structures are shown in ▶ **Fig. 5**.

The antagonism of P1-3 against the NK_1 receptor was examined with the calcium assay to assess the potency suppressing cytosolic calcium fluorescence. The maximum fluorescent unit was induced by the minimum concentration of substance P at 3.29 nM, and it dose-dependently decreased with pretreated P1, P2, and P3, demonstrating that they were favorably potent compared to the known antagonist L-703,606 oxalate (▶ **Fig. 6**). The antagonistic activities tended to decrease from P1 to P3; the IC_{50} values of P1-3 and L-703,606 oxalate were shown at 0.5, 1.3, 1.7, and 2.8 µM, respectively. It could be suggested that *cis*-coumaroyl was more advantageous than *trans*-coumaroyl for preoccupying the binding site on the NK_1 receptor. The antagonistic activity of P1, 4 *cis*-coumaroyl moiety-conjugated thermospermine, and spermine, showing the strongest potency among the isomers, was visually represented by the real-time monitoring system (Supporting Information, video file). P1 suppressed substance P-induced calcium release compared to the selective NK_1 receptor antagonist L-703,606. The emission indicating the calcium release induced by substance P was dramatically inhibited by the treatment of 0.49 and 6.19 µM P1. In addition, it was reported that substance P and NK_1 receptors were relatively overexpressed, which activated EGFR and HER2-related signal transduction, modulating further proliferation of the breast cancer cells [20]. To examine whether P1, P2, and P3 possibly inhibited substance P-induced proliferation as the antagonists, HER2-positive (MDA-MB-453; ER-, PR-, HER2 +) and HER2-negative (MDA-MB-231; ER-, PR-, HER2-) breast cancer cell lines were compared. The 5 nM of substance P selectively activated the proliferation up to 120 % in MDA-MB-453, but the substance P-dependent prolif-



▶ **Fig. 3** HPLC chromatograms and UV spectra of P1, P2, and P3 separated by CCC. The hydroxycinnamates-conjugated polyamines exhibited different UV spectra that the λ_{max} moved to a longer wavelength from P1 to P3. The result implied their structural differences were possibly derived from the variation of *cis*/*trans* hydroxycinnamate groups conjugated on aliphatic tetraamine.



▶ **Fig. 4** Structural differences in the 2 polyamines and the unique mass fragment of thermospermine. The asymmetric aliphatic carbon arrangement leads thermospermine to have a unique MS^2 fragment of *m*/*z* 144, which can be a marker to distinguish conjugated thermospermines from spermine.

▶ **Table 2** ^{1}H NMR data of compounds **1a**-**3b** (δ in ppm, J in Hz, 600 MHz).

Position	1_a	1_b	2_a	2_b	3_a	3_b
OH'	9.66, br s	9.66, br s	9.91, br s	9.91, br s	9.82, br s	9.82, br s
OH''	9.66, br s	9.66, br s	9.75, br s	9.75, br s	9.83, br s	9.83, br s
OH'''	9.66, br s	9.66, br s	9.75, br s	9.75, br s	9.84, br s	9.84, br s
OH''''	9.66, br s	9.66, br s	9.91, br s	9.91, br s	9.85, br s	9.85, br s
1-NH	7.98, m	7.96, m	7.97, m	7.95, m	7.97, m	7.97, m
14-NH	8.05, m	8.03, m	8.04, m	8.01, m	8.09, m	8.09, m
3'	6.49, d (12.7)	6.51, d (12.7)	7.33, d (15.7)	7.30, d (15.7)	7.34, d (15.7)	7.31, d (15.7)
3''	6.38, d (12.7)	6.38, d (12.7)	6.38, d (12.6)	6.39, d (12.6)	7.41, d (15.7)	7.40, d (15.7)
3'''	6.45, d (12.8)	6.40, d (12.8)	6.45, d (12.6)	6.41, d (12.6)	7.41, d (15.7)	7.40, d (15.7)
3''''	6.48, d (12.8)	6.50, d (12.8)	7.33, d (15.7)	7.30, d (15.7)	7.34, d (15.7)	7.31, d (15.7)
5', 9'	7.60, m	7.61, m	7.38, d (8.7)	7.38, d (8.7)	7.56, d (8.5)	7.39, d (8.4)
5'', 9''	7.18, d (8.5)	7.19, d (8.5)	7.18, d (8.7)	7.18, d (8.7)	7.49, d (8.5)	7.38, d (8.4)
5''', 9'''	7.23, d (8.5)	7.21, d (8.5)	7.22, d (8.7)	7.20, d (8.7)	7.46, d (8.5)	7.38, d (8.4)
5'''', 9''''	7.60, m	7.61, m	7.38, d (8.7)	7.38, d (8.7)	7.52, d (8.5)	7.39, d (8.4)
2'	5.79, d (12.8)	5.78, d (12.8)	6.41, d (15.7)	6.36, d (15.7)	6.42, d (15.7)	6.40, d (15.7)
2''	5.83, d (12.8)	5.87, d (12.8)	5.84, d (12.6)	5.89, d (12.6)	6.92, d (15.7)	6.83, d (15.7)
2'''	5.95, d (12.8)	5.92, d (12.8)	5.95, d (12.6)	5.93, d (12.6)	6.90, d (15.7)	6.82, d (15.7)
2''''	5.75, d (12.8)	5.74, d (12.8)	6.40, d (15.7)	6.36, d (15.7)	6.41, d (15.7)	6.40, d (15.7)
6', 8'	6.68, m	6.68, m	6.78, m	6.78, m	6.77, d (8.6)	6.79, d (8.6)
6'', 8''	6.70, m	6.70, m	6.69, m	6.69, m	6.71, d (8.4)	6.79, d (8.6)
6''', 8'''	6.70, m	6.70, m	6.69, m	6.69, m	6.7, d (8.4)	6.79, d (8.6)
6'''', 8''''	6.68, m	6.68, m	6.78, m	6.78, m	6.76, d (8.6)	6.79, d (8.6)
2	2.99, m	3.11, m	3.04, m	3.16, m	3.16, m	3.16, m
3	1.53, m	1.64, m	1.54, m	1.65, m	1.68, m	1.68, m
4	3.15, m	3.23, m	3.16, m	3.25, m	3.46, m	3.49, m
6	3.20, m	3.23, m	3.22, m	3.25, m	3.35, m	3.36, m
7	1.35, m	1.35, m	1.37, m	1.37, m	1.48, m	1.60, m
8	3.33, m	1.16, m	3.35, m	1.18, m	3.39, m	1.51, m
9		3.06, m		3.07, m		3.36, m
10	3.24, m		3.25, m		3.46, m	
11	1.50, m	3.32, m	1.52, m	3.35, m	1.56, m	3.49, m
12	1.58, m	1.70, m	1.58, m	1.71, m	1.75, m	1.75, m
13	3.02, m	3.14, m	3.07, m	3.19, m	3.21, m	3.21, m

■[a] Assignments were based on HSQC and HMBC experiments. Compounds **1a**-**3b** were measured in DMSO-d_6

eration was not observed in MDA-MB-231 (▶ **Fig. 7**). The antagonistic activities of P1-3 that downregulated substance P/NK_1 receptor-induced proliferation were consequentially shown in MDA-MB-453 only. The increased proliferation rate by substance P was recovered to the blank state with the treatment of P1-3, exhibiting potent proliferative inhibition compared to L-703,606 oxalate (P1, IC_{50} = 2.6 µM; P2, IC_{50} = 5.4 µM; P3, IC_{50} = 6.7 µM; L-703,606 oxalate, IC_{50} = 3.2 µM). Taken together, it was demonstrated that the isomers of TCS were potent antagonists of the NK_1 receptor and were applicable for the treatment of substance P/NK_1 receptor-related disease, including HER2-positive cancers, as well as pain, mood disorders, and insomnia.

Materials and Methods

General experimental procedures

Mitsubishi Chemical Corporation Diaion HP-20 and GE Healthcare Life Sciences Sephadex LH-20 were used for open-column chromatography, and TLC was performed using 60 F_{254} silica gel-coated EMD Millipore plates. HPLC/MS analysis was performed with an Agilent Technologies HPLC 1100 series with a G1322A degasser, a G1311A quaternary pump, a G1313A autosampler, a G1316A column oven, and a G1315A diode array detector with a Zorbax SB-Aq C_{18} column (3.5 µm, i.d. 4.6 × 150 mm and i.d. 4.6 × 75 mm) and a Thermo Fisher Scientific Finnigan LCQ Deca MS system. The mobile phases were composed of water with 0.1 % formic acid (A) and J.T. Baker Chemical Co. HPLC grade methanol with 0.1 % formic acid (B). The flow rate was set to 0.3 mL/min. The running program was initiated with 25 % B for 2 min, then 25–45 % B for 5 min, and the gradient was further increased from 45–85 % for 33 min. Mass spec-

▶ **Table 3** ^{13}C NMR data of compounds **1a**-**3b** (δ in ppm, J in Hz, 150 MHz).

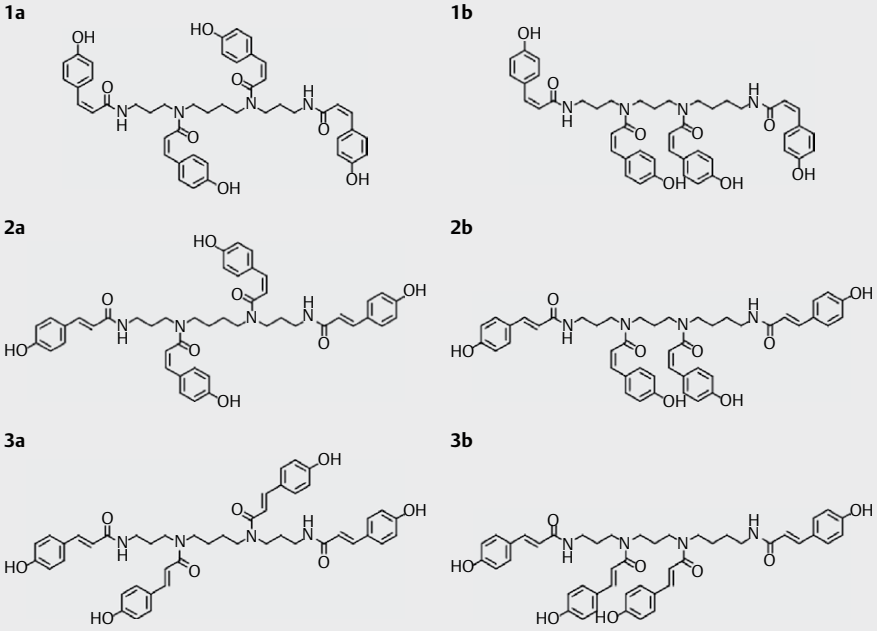
Position	1a[a]	1b[a]	2a[a]	2b[a]	3a[a]	3b[a]
1'	166.2, C	166.2, C	165.4, C	165.4, C	165.5, C	165.3, C
1''	168.1, C	168.0, C	168.5, C	168.1, C	165.6, C	165.3, C
1'''	168.2, C	168.1, C	168.5, C	168.2, C	165.6, C	165.3, C
1''''	166.2, C	166.2, C	165.4, C	165.4, C	165.5, C	165.3, C
7'	157.8, C	157.8, C	158.9, C	158.9, C	158.8, C	158.8, C
7''	157.6, C	157.6, C	157.7, C	157.7, C	158.8, C	158.8, C
7'''	157.6, C	157.6, C	157.7, C	157.7, C	158.8, C	158.8, C
7''''	157.8, C	157.8, C	158.9, C	158.9, C	158.8, C	158.8, C
3'	136.5, CH	136.4, CH	138.7, CH	138.7, CH	138.8, CH	138.6, CH
3''	131.9, CH	131.9, CH	131.9, CH	131.9, CH	141.5, CH	141.5, CH
3'''	131.9, CH	132.0, CH	131.9, CH	131.9, CH	141.5, CH	141.5, CH
3''''	136.5, CH	136.4, CH	138.7, CH	138.7, CH	138.8, CH	138.6, CH
5', 9'	131.9, CH_2	131.9, CH_2	129.3, CH_2	129.3, CH_2	129.8, CH_2	129.2, CH_2
5'', 9''	129.9, CH_2	129.9, CH_2	129.9, CH_2	129.9, CH_2	129.7, CH_2	129.2, CH_2
5''', 9'''	123.0, CH_2	123.0, CH_2	123.0, CH_2	123.0, CH_2	129.7, CH_2	129.2, CH_2
5'''', 9''''	131.9, CH_2	131.9, CH_2	129.3, CH_2	129.3, CH_2	129.8, CH_2	129.2, CH_2
4'	126.3, C	126.3, C	125.8, C	125.9, C	125.8, C	125.9, C
4''	126.4, C	126.4, C	126.4, C	126.5, C	126.2, C	126.1, C
4'''	126.5, C	126.4, C	126.4, C	126.5, C	126.2, C	126.1, C
4''''	126.3, C	126.3, C	125.8, C	125.9, C	125.8, C	125.9, C
2'	120.9, CH	120.9, CH	118.7, CH	118.5, CH	118.4, CH	118.7, CH
2''	121.0, CH	120.8, CH	121.1, CH	121.1, CH	114.7, CH	114.6, CH
2'''	120.0, CH	120.9, CH	121.1, CH	121.1, CH	114.7, CH	114.6, CH
2''''	120.7, CH	120.7, CH	118.7, CH	118.5, CH	118.4, CH	118.7, CH
6', 8'	115.2, CH_2	115.2, CH_2	115.8, CH_2	115.8, CH_2	115.7, CH_2	115.7, CH_2
6'', 8''	114.7, CH_2	114.7, CH_2	115.2, CH_2	115.2, CH_2	115.6, CH_2	115.7, CH_2
6''', 8'''	114.7, CH_2	114.7, CH_2	115.7, CH_2	115.7, CH_2	115.6, CH_2	115.7, CH_2
6'''', 8''''	115.2, CH_2	115.2, CH_2	115.8, CH_2	115.8, CH_2	115.7 CH_2	115.7, CH_2
2	36.0, CH_2	36.5, CH_2	36.2, CH_2	36.7, CH_2	36.6, CH_2	36.6, CH_2
3	28.3, CH_2	27.0, CH_2	28.6, CH_2	27.3, CH_2	27.9, CH_2	27.9, CH_2
4	45.5, CH_2	42.1, CH_2	45.4, CH_2	42.1, CH_2	44.9, CH_2	46.9, CH_2
6	43.6, CH_2	47.5, CH_2	43.6, CH_2	47.6, CH_2	45.5, CH_2	43.7, CH_2
7	24.0, CH_2	25.8, CH_2	24.1, CH_2	25.8, CH_2	25.0, CH_2	26.7, CH_2
8	43.9, CH_2	25.5, CH_2	43.9, CH_2	25.5, CH_2	45.1, CH_2	26.7, CH_2
9		47.4, CH_2		47.4, CH_2		43.7, CH_2
10	45.6, CH_2		45.8, CH_2		44.8, CH_2	
11	24.5, CH_2	42.2, CH_2	24.5, CH_2	42.3, CH_2	24.8, CH_2	46.9, CH_2
12	28.3, CH_2	27.0, CH_2	28.6, CH_2	27.3, CH_2	29.7, CH_2	29.7, CH_2
13	36.0, CH_2	36.5, CH_2	36.2, CH_2	36.7, CH_2	36.2, CH_2	36.2, CH_2

[a] Assignments were based on HSQC and HMBC experiments. Compounds **1a**-**3b** were measured in DMSO-d_6

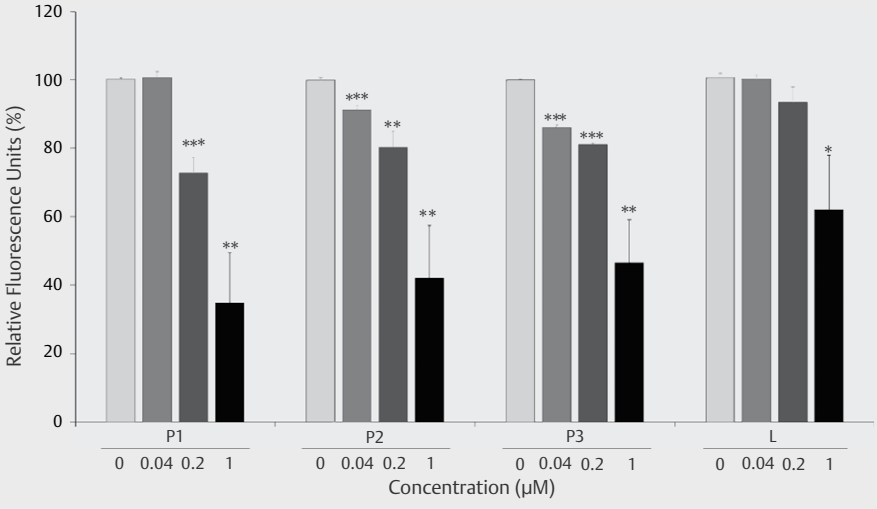
tra were obtained by electrospray ionization in both the negative and positive ionization modes at the range of m/z 100–1 000, and the analysis was conducted using the following conditions: spray voltage, 5.0 kV; sheath gas flow rate, 60 arb; auxiliary gas flow rate, 9.3 arb; capillary voltage, 45.0 V; capillary temperature, 290 °C; and tube lens, 20 V. The NMR spectra were recorded on a Bruker AVANCE 600 spectrometer operated at 600 MHz for ^1H and at 150 MHz for ^{13}C.

The preparative CCC separation was performed using a Tauto Biotechnique Company TBE-300A CCC equipped with a 280-mL coil column composed of polytetrafluoroethylene tubing, including a 20-mL sample loop with the following components: a Hitachi L-6200A Intelligent Pump, a Sedere SEDEX 60 LT ELSD, a Sungchang Electrics automatic voltage regulator, an Amersham Biosciences circulator, an Advantec MFS SF-2120 super fraction collector, and a Younglin Instrument Autochro data module with Autochro-2000 1.0 software.

EMD Millipore Ready-to-Assay™ NK_1 tachykinin receptor frozen cells, Sigma-Aldrich substance P acetate salt hydrate, Santa Cruz Biotechnology L-703,606 oxalate, Abcam Fluo-8 AM, TCI America



▶ **Fig. 5** Structures of tetra-*cis*/*trans*-coumaroyl polyamines **1a-3b** isolated from *M. chamomilla*.

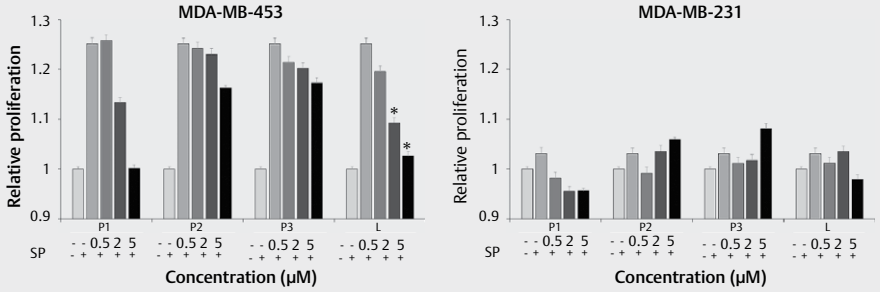


▶ **Fig. 6** Antagonistic activity of P1-3 assessed by the calcium assay. The statistical analyses were performed by 2-way ANOVA with replication, followed by Dunnett's test. * $p<0.05$, * * $p<0.01$, and * * * $p<0.001$ indicated significant differences from the antagonistic activity of P1-3, suppressing the calcium fluorescence induced by substance P (EC_{90} 3.29 nM).

probenecid, and Corning Inc. black 96-well clear bottom plates were used for the calcium assay. The purity of the L-703,606 oxalate was greater than 96 %. The assay was conducted using a Molecular Devices SpectraMax M5 multiplate reader, and the real-time images were obtained with a PerkinElmer Operetta with Harmony® high-content imaging software.

MDA-MB-231 and MDA-MB-453 human breast cancer cells were obtained from the Korea Cell Bank. Dulbecco's phosphate-buffered saline (DPBS), DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and substance P acetate salt hydrate were purchased from Sigma-Aldrich. DMEM (high-glucose) and fetal bovine serum (FBS) were obtained from GenDepot. Cell viability assessed by the MTT assay was measured using a Molecular Devices Emax Microplate Reader.

Plant material



▶ **Fig. 7** Suppressing activity of P1-3 on the substance P-induced proliferation in MDA-MB-453 and MDA-MB-231. Substance P selectively induced the proliferation of HER2-positive (MDA-MB-453; ER-, PR-, HER2 +) breast cancer cells up to 120 percent, while the proliferation rate in HER2-negative (MDA-MB-231; ER-, PR-, HER2-) breast cancer cells was not relatively affected by substance P treatment. The significant differences between the substance P non-treated and treated group were calculated by Dunnett's test (* $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$).

Dried German chamomile flowers were purchased from Agricultural Corporation Namwonherb Co., Ltd. A voucher specimen (SNU-14-517) is deposited in the herbarium of the Natural Products Research Institute (NPRI), College of Pharmacy, Seoul National University. The specimen was identified as *M. chamomilla* by Professor Young Bae Suh at the College of Pharmacy, Seoul National University.

Extraction and isolation

5 kg of dried chamomile flower were soaked in 15 L of methanol for 24 h at room temperature. The methanolic extract was concentrated using a rotary vacuum evaporator at 45 °C, and ethyl acetate was obtained by solvent partitioning. Next, 3 L of Diaion HP-20 resin were activated with the same volume of methanol in a 5-L bucket, and the resin was equilibrated in 50 % aqueous methanol. The constituents in the dried ethyl acetate sample were adsorbed into the resin for 2 h, and then the resin was washed with 70 % methanol. The 90 % methanol fraction was collected, then further subfractionated through Sephadex LH-20 column chromatography with 80 % MeOH. The enriched fractions were selected by silica gel thin-layer chromatography with the solvent composition of ethyl acetate-MeOH-water (10:1:0.5, v/v/v); the R_f was 0.33 under UV 254 nm. Methylene chloride-MeOH-water (1:1:1, v/v/v) was selected as the solvent system, and the aqueous upper phase was used as the stationary phase. The tubing column within the CCC was entirely filled with the stationary phase at a flow rate of 9 mL/min. The operation was then performed at a rotation speed of 850 rpm, with a circulator temperature of 25 °C, connecting the outlet through a split valve to the ELSD system at 35 °C, a gain of 4, and nitrogen gas pressure at 2.5 bar. A 100-mg sample was dissolved in 6 mL of the lower and upper phases (1:1, v/v), and was injected when the mobile phase began to be pumped out of the column at a flow rate of 0.8 mL/min.

Calcium assay

The antagonistic activities of TCTS and TCS for suppressing substance P binding to NK_1 receptors were evaluated by a calcium assay using Ready-to-Assay™ NK_1 tachykinin receptor frozen cells, following the assay steps as suggested in the manufacturer's protocol. The excitation and emission wavelengths were set at 488 and 515 nm, respectively, and the known NK_1 receptor antagonist L-703,606 oxalate was used as the positive control. The relative increase in the intracellular calcium fluorescence was calculated by the following formula (abbreviations: Max F, maximum fluorescence; B, buffer solution-treated (blank); C, compound treated with substance P; S, substance P-treated only):

$$\frac{\text{Max F}^{C} - \text{Max F}^{B}}{\text{Max F}^{S} - \text{Max F}^{B}} \times 100\,(\%)$$

Antiproliferation assay

Cultures were maintained in DMEM supplemented with 10 % FBS and antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL) in a humidified atmosphere incubator at 37 °C with 5 % CO_2. The toxicity and antiproliferative activity of the compounds were evaluated by cell viability with the MTT assay. MDA-MB-453 and MDA-MB-231 breast cancer cells were seeded into 96-well plates at densities of 3×10^4 and 1×10^4 cells per well, respectively. The plates were maintained at 37 °C for 24 h and the media in the plate was exchanged with FBS-excluded DMEM with the target compounds or the positive control L-703,606 oxalate. MDA-MB-453 was further incubated for 50 h and MDA-MB-231 for 24 h, considering their doubling times. The MTT solution (0.5 mg/mL) was added to each well, and the cells were incubated for another 2 h. The cell viability was evaluated by measuring the absorbance at a 540 nm wavelength using a microplate reader. The relative proliferation factors were calculated by the following formula:

$$\frac{\text{Mean absorbance of sample treated group}}{\text{Mean absorbance of blank group}}$$

Statistical analysis

All data are presented as the mean ± standard deviation (SD) of n = 3 independent experiments, and each concentration was performed in triplicate. Statistical analysis was performed using Microsoft Excel 2013, and the significant differences between the control and experimental groups were calculated by 2-way ANOVA with replication, followed by Dunnett's test (* $p < 0.05$, * * $p < 0.01$, * * * $p < 0.001$).

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Conflicts of Interest

The authors declare no competing financial interest.

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