Journal of Food and Drug Analysis, Vol. 16, No. 3, 2008, Pages 26-30 藥物食品分析 第十六卷 第三期

Norepinephrine Transporter Inhibitors from *Polygala tenuifolia*

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(Received: March 22, 2007; Accepted: November 4, 2007)

ABSTRACT

Using norepinephrine transporter binding assay-directed fractionation, polygalasaponin XXVIII (**1**) and 3-*O*-β-D-glucopyranosyl presenegenin 28-[*O*-β-D-galatopyranosyl(1→4)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl(1→2)-β-D-fucopyranosyl] ester (**2**), together with five xanthone glycosides, neolancerin (**3**), polygalaxanthone IX (**4**), sibiricaxanthone (**5**), polygalaxanthone III (**6**), and 7-*O*-methylmangiferin (**7**), five phenolic glycosides, tenuifoliside A (**8**), sibiricoses A_3 (**9**), A_5 (**10**), A_6 (**11**) and 3',6-disinapoyl sucrose (**12**), a triterpenoid, tenuifolin (**13**), methyl sinapoate (**14**), and adenosine (**15**) were isolated from the active fraction of an aqueous extract of the roots of *Polygala tenuifolia*. Their structures were elucidated by extensive NMR and mass spectroscopic analyses. Compounds **1** and **2** were shown to inhibit norepinephrine reuptake through blocking norepinephrine transport by 77% and 28% in the norepinephrine transporter binding assay at concentrations of 4.53 μM and 3.95 μM, respectively.

Key words: *Polygala tenuifolia*, norepinephrine transporter inhibitor, saponins, xanthone glycosides, phenolic glycosides

INTRODUCTION

Polygala tenuifolia (Polygalaceae) is a perennial herb and widely distributed in Mainland China. Its roots (Yuan Zhi) have been used in the traditional Chinese medicine as an expectorant, sedative, and tranquilizing agent and also for insomnia, neurasthenia, restlessness etc[1]. Saponins[2,3], xanthones[4-8], and phenolic glycosides[9-15] have been reported from the titled plant. Many studies were performed to investigate its role in preventing neuronal damage. For example, an ethanolic extract and n-butanol fraction of *P. tenuifolia* reduced brain damage caused by ischemia and reperfusion[16]. Moreover, the extract of *P. tenuifolia* prevented NMDA-induced neuronal cell damage[17]. Tenuigenin, a triterpene component isolated from *P. tenuifolia*, inhibited the secretion of amyloid β protein in cultured cells[18]. The reduction in cholinergic function by *P. tenuifolia* prevented cocaine-induced behavioral effects *via* the activation of the adenosine A_{2A} receptor[19]. Similarly, an aqueous extract of *P. tenuifolia* exerted repairing effects on the memory and behavioral disorders in rats[20]. In addition, acylated oligosaccharides[21] and phenolic components[22] exhibited cognitive improving, cerebral protection and anti-oxidative effects. Norepinephrine reuptake inhibitors such as venlafaxine and bupropion have been demonstrated to exert their actions through multiple receptors within the CNS[23,24]. In order to further investigate its active neural components, we describe herein the bioassay guided isolation and structural elucidation of active principles from the bioactive fraction of an aqueous extract of Yuan Zhi using blocking norepinephrine transporter assay.

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MATERIALS AND METHODS

Instruments and Materials

Melting points were measured on a Yanagimoto micromelting point apparatus and were uncorrected. IR spectra were recorded on a Nicolet avatar 320 FT-IR spectrophotometer. NMR experiments were performed on a Varian unity INOVA-500 spectrometer. Mass spectra (FAB-MS and HR-FAB-MS) were taken on a JEOL JMS-HX110 and a JEOL SX-102A mass spectrometer, respectively. Optical rotations were run on a JASCO DIP-370 polarimeter. Column chromatography was performed on RP-18 (Merck) and Sephadex LH-20 (Pharmacia) columns.

II. *Plant Material*

The roots of *P. tenuifolia* WILLD. were imported by Jun-Chi Co. directly from mainland China, in May 2003 and authenticated by Medical and Pharmaceutical Indus-

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try Technology and Development Center (MPITDC), Taipei County, Taiwan. A voucher specimen (No. 920021) was deposited at the herbarium of MPITDC.

III. *Extraction and Isolation*

The air-dried and powdered roots (1.25 kg) of *P. tenuifolia* were extracted with water (5 L, 2 hr) under reflux. The water extract (152 g) was subjected to Diaion HP-20 column chromatography (2.0 kg) and eluated with H_2O (45 L), 50% aq. MeOH (30 L) and MeOH (25 L), successively. The bioactive 50% aq MeOH eluate (44 g) was rechromatographed on Sephadex LH-20 column (1.0 L) using a gradient elution of 80% aq. MeOH to MeOH. The selected active fractions (50% MeOH) (3.0 g) were rechromatographed over Sephadex LH-20 column (300

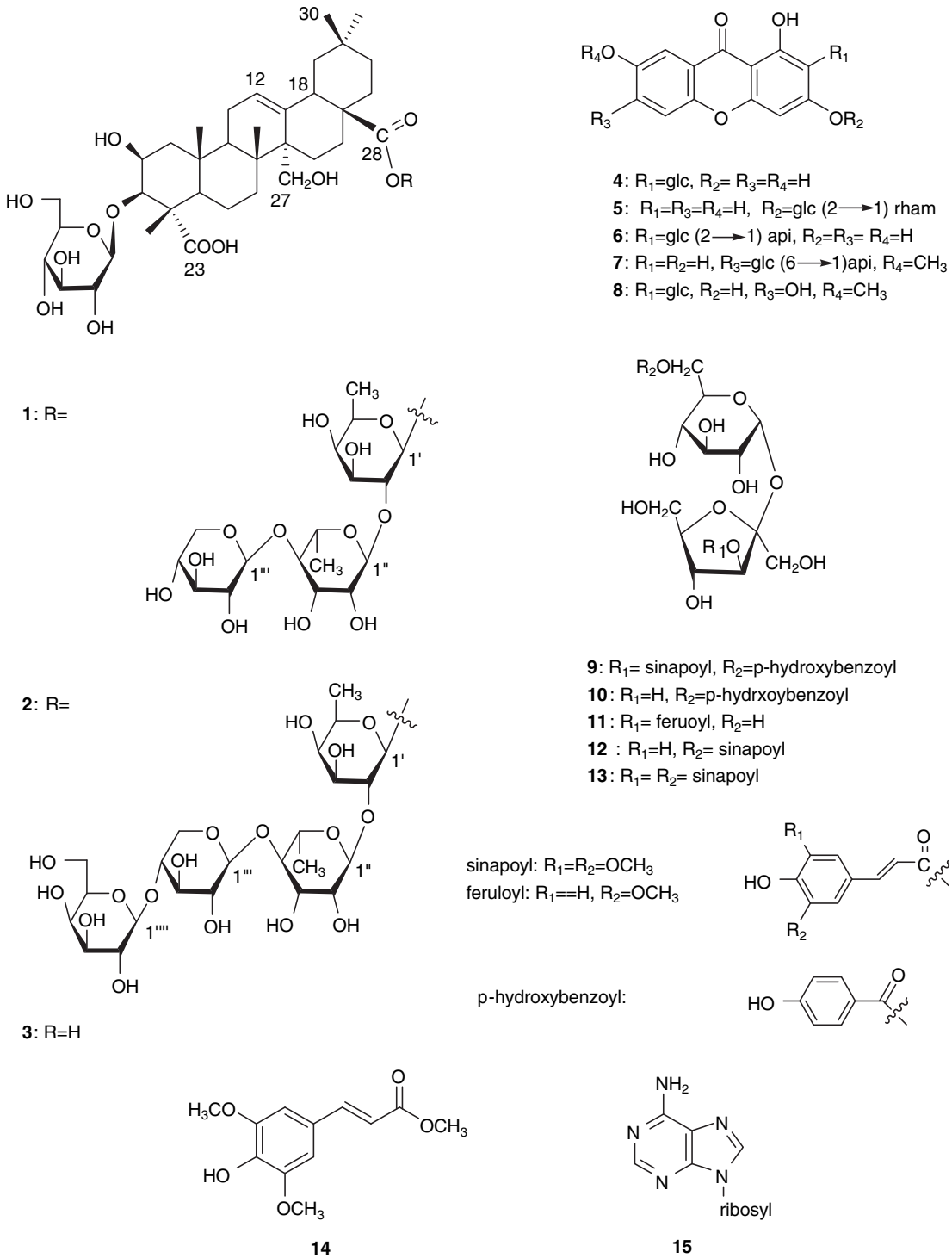

Figure 1. The structures of the compounds isolated from the active fraction of an aqueous extract of the roots of *Polygala tenuifolia*.

mL) using 80% aq. MeOH and MeOH as eluents to yield five fractions. Each fraction was further purified on a Sephadex LH-20 (80% aq. MeOH ~100% MeOH) and RP-18 (40%~60% aq. MeOH) columns repeatedly to afford **1** (28 mg), **2** (46 mg), **3** (10 mg), **4** (15 mg), **5** (12 mg), **6** (18 mg), **7** (25 mg), **8** (35 mg), **9** (11 mg), **10** (8 mg), and **11** (12 mg), **12** (65 mg), **13** (15 mg), **14** (12 mg), and **15** (35 mg).

(I) *Compound 1*[25]

Colorless amorphous powder; $[\alpha]_D$: –10° (*c* 0.3, MeOH); IR υ_{max} (KBr) cm^{-1}: 3450-3300, 2938, 1740, 1708, 1630, 1446, 1383, 1262, 1167, 1057; ^1H NMR (500 MHz, C_5D_5N): δ 0.72, 0.85, 1.07, 1.50 and 1.89 (3H each, s), 1.43 and 1.61 (3H each, d, *J* = 6.5 Hz, fuc-6-H and rha-H-6), 3.15 (1H, dd, *J* = 10.5, 3.0 Hz, H-18), 3.73 and 4.02 (1H each, d, *J* = 12.0 Hz, H-27), 4.97 (1H, d, *J* = 7.0 Hz, xyl-H-1), 5.20 (1H, d, *J* = 7.5 Hz, glc-H-1), 5.75 (1H, t, *J* = 4.0 Hz, H-12), 6.00 (1H, d, *J* = 8.0 Hz, fuc-H-1), 6.30 (1H, br s, rha-H-1); ^{13}C NMR (125 MHz, C_5D_5N): δ 14.2 (q, C-24), 16.9 (q, fuc-C-6), 17.4 (q, C-25), 18.5 (q, rha-C-6), 18.7 (q, C-26), 21.3 (t, C-6), 23.8 (t, C-11), 24.0 (t, C-15), 23.9 (q, C-30), 24.4 (t, C-16), 30.7 (s, C-20), 32.3 (t, C-7), 33.0 (q, C-29), 33.6 (t, C-22), 33.7 (t, C-21), 36.9 (s, C-10), 41.1 (s, C-8), 41.9 (d, C-18), 44.2 (t, C-1), 45.3 (t, C-19), 46.8 (s, C-17), 47.9 (s, C-14), 49.2 (d, C-9), 52.5 (d, C-5), 52.9 (s, C-4), 62.5 (t, glc-C-6), 64.3 (t, C-27), 67.3 (t, xyl-C-5), 68.2 (d, rha-C-5), 70.2 (d, xyl-C-4), 70.8 (d, glc-C-4), 71.4 (d, C-2), 71.7 (d, rha-C-2), 72.4 (d, fuc-C-5), 72.4 (d, rha-C-3), 73.1 (d, fuc-C-4), 73.7 (d, fuc-C-3), 75.2 (d, glc-C-2), 76.1 (d, fuc-C-2),76.6 (d, xyl-C-2), 78.2 (d, glc-C-5), 78.3 (d, glc-C-3), 78.6 (d, xyl-C-3), 84.9 (d, rha-C-4), 86.0 (d, C-3), 94.7 (d, fuc-C-1), 101.2 (d, rha-C-1), 105.2 (d, glc-C-1), 107.2 (d, xyl-C-1), 127.7 (d, C-12), 138.9 (s, C-13), 176.7 (s, C-23), 180.7 (s, C-28); FAB-MS *m/z*: 1105 $[M+H]^+$ (10), 412 (100); HR-FAB-MS *m/z*: 1105.5461 (calculated for $C_{53}H_{85}O_{24}$, 1105.5432).

(II) *Compound 2*[26]

Colorless amorphous powder; $[\alpha]_D$: +33° (*c* 0.3, MeOH); IR υ_{max} (KBr) cm^{-1}: 3500-3300, 1923, 1750, 1719, 1643, 1453, 1386, 1073, 950, 899; ^1H-NMR (500 MHz, C_5D_5N): δ 0.76, 0.91, 1.13, 1.53 and 1.99 (3H each, s), 1.48 and 1.63 (3H each, d, *J* = 6.5 Hz, fuc-H-6 and rha-H-6), 3.21 (1H, dd, *J* = 10.0, 3.0 Hz, H-18), 4.95 (1H, d, *J* = 7.5 Hz, gal-H-1), 4.98 (1H, d, *J* = 7.5 Hz, xyl-1-H), 5.07 (1H, d, *J* = 8.0 Hz, glc-H-1), 5.79 (1H, t, *J* = 4.0 Hz, H-12), 6.06 (1H, d, *J* = 8.0 Hz, fuc-H-1), 6.40 (1H, br s, rha-H-1); ^{13}C NMR (125 MHz, C_5D_5N): δ 14.2 (q, C-24), 16.9 (q, fuc-C-6), 17.5 (q, C-25), 18.5 (q, rha-C-6), 18.5 (q, C-26), 21.8 (t, C-6), 23.9 (t, C-11), 23.8 (t, C-15), 23.9 (q, C-30), 24.3 (t, C-16), 30.7 (s, C-20), 32.8 (t, C-7), 33.0 (q, C-29), 33.6 (t, C-22), 33.8 (t, C-21), 37.0 (s, C-10), 41.1 (s, C-8), 41.9 (d, C-18), 44.3 (t, C-1), 45.3 (t, C-19), 46.8 (s, C-17), 48.0 (s, C-14), 49.3 (d, C-9), 52.5 (d, C-5), 52.8 (s, C-4), 62.2 (t, gal-C-6), 62.7 (t, glc-C-6), 64.3 (t, C-27), 65.4 (t, xyl-C-5), 68.1 (d, rha-C-5), 70.1 (d, gal-C-4), 70.2 (d, xyl-C-4), 70.3 (d, glc-C-4), 70.3 (d, C-2), 71.7 (d, rha-C-2), 72.4 (d, rha-C-3), 72.5 (d, gal-C-2), 72.5 (d, fuc-C-5), 73.2 (d, fuc-C-4), 74.1 (d, fuc-C-3), 75.0 (xyl-C-2), 75.0 (d, gal-C-3), 75.2 (d, glc-2), 75.7 (xyl-C-3), 76.6 (d, gal-C-5), 77.3 (d, fuc-C-2), 78.3 (d, glc-C-5), 78.3 (d, glc-C-3), 78.3 (d, xyl-C-4), 85.2 (d, rha-C-4), 85.9 (d, C-3), 94.7 (d, fuc-C-1), 101.3 (d, rha-C-1), 104.5 (d, gal-C-1)105.4 (d, glc-C-1), 106.9 (d, xyl-C-1), 127.8 (d, C-12), 138.9 (s, C-13), 176.7 (s, C-23), 180.7 (s, C-28), FAB-MS *m/z*: 1267 $[M+H]^+$ (8), 412 (100); HR-FAB-MS *m/z*: 1267.5943 (calculated for $C_{59}H_{95}O_{29}$, 1267.5960).

IV. *Acid Hydrolysis of 1 and 2*

Individually, each compound (5 mg) was hydrolyzed with 2 N aqueous CF_3COOH (3 mL) in water bath for 2 hr[9]. After evaporation, the reaction mixture was diluted with H_2O (10 mL) and extracted with ethyl acetate (EtOAc, 10 mL). The combined EtOAc extract was washed with H_2O and evaporated to afford the aglycone presenegenin[2]. The aqueous layer was neutralized with sodium bicarbonate and evaporated to dryness. The residue was analyzed and compared with authentic samples by TLC using $CHCl_3$:MeOH:H_2O (7:3:0.5, v/v) as developing solvent, and yield xylose (*Rf* 0.25), rhamnose (*Rf* 0.40), fucose (*Rf* 0.36) and glucose (*Rf* 0.15) for 1, and give galactose (*Rf* 0.13), xylose, rhamnose, fucose and glucose for **2**, respectively. After the sugar mixtures of **1** and **2** were evaporated, respectively, pyridine (30 μL), hexamethyldisilazane (30 μL), and trimethylsilyl chloride (30 μL) was added to each residue. The reaction mixture was heated at 60°C for 30 min. The supernatant was then subjected to gas chromatrgraphy (GC) analysis. GC conditions were: column, Supelco SPB^{TM}-1 capillary column (30 m × 0.25 mm × 0.25 μm), carrier gas, He. The retention times (t_R) are: per-trimethylsilyl glucose, 9.30 min, per-trimethylsilyl galactose, 9.15 min, per-trimethylsilyl fucose, 7.5 min, per-trimethylsilyl rhamnose, 8.10 min, and per-trimethylsilyl xylose, 8.09 min.

V. *Alkaline Hydrolysis of 1 and 2*

Each saponin (4 mg) was refluxed at 100°C in a water bath with 5% aqueous KOH solution (5 mL) for 1 hr. The mixture was neutralized with dilute HCl and then extracted with butanol (BuOH). The combined BuOH extracts were washed with H_2O and evaporated to afford tenuifolin which was identified mainly by NMR and by comparison with literature values[2].

VI. *Membrane Binding Assay*

Membranes from dog kidney MDCK cells, that were stably transfected with the human norepinephrine transporter, were used. Total cell membranes were prepared from transfected cells grown to confluence in 500 cm^2

tissue culture dishes. Cells were scraped into a centrifuge tube and pelleted at 900 ×g and 4°C for 10 min. The cell pellet was resuspended in modified Tri-HCl buffer (50 mM Tris-HCl, 100 mM NaCl, 1 μM leupeplin, 10 μM PMSF, pH 7.4), and centrifuged at 17000 ×g and 4°C for 30 min. The pellet was resuspended, homogenized using a glass homogenizer with a Teflon pestle and centrifuged at 17000 ×g and 4°C for 90 min. Pellet was collected and resuspended in modified Tris-HCl buffer. Protein concentrations were determined using BCA protein assay reagent (Pierce, USA). For the binding assay, a 40 μg aliquot of membrane protein was incubated with 0.2 nM [^{125}I]RTI-55 at 4°C for 3 hr. The binding was terminated by rapid vacuum filtration over Whatman GF/B filters soaked in 0.3% polyethylinemine followed by three rapid 1 mL of washes in ice-cold modified Tris-HCl buffer. Bound radioactivity was measured by gamma emission spectrometry. Non-specific binding was determined using 10 μM desipramine as positive control and was subtracted from the data to yield the specific binding[27].

RESULTS AND DISCUSSION

Under norepinephrine transporter binding assay-directed fractionation, 15 compounds were isolated by normal- and reverse-phase column chromatography. Their structures were identified by spectroscopic methods, including 1D- and 2D-NMR, IR and MS, and by comparison with the literature data. These compounds included two active saponins, polygalasaponin XXVIII (**1**)[25] and 3-*O*-β-D-glucopyranosyl presenegenin28-[*O*-β-D-galato-pyranosyl(1→4)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl(1→2)-β-D-fucopyranosyl] ester (**2**)[26], and tenuifolin (**3**)[2], together with five known xanthones, neolancerin (**4**)[28] and polygalaxanthone IX (**5**)[8], sibiricaxanthone B (**6**)[29], polygalaxanthone III (**7**)[6,29] and 7-*O*-methylmangiferin (**8**)[30], five known phenolic glycosides, tenuifoliside A (**9**)[10,29], sibiricoses A_3 (**10**)[29], A_5 (**11**)[29], A_6 (**12**)[29] and 3',6-disinapoyl sucrose (**13**)[10], methyl sinapoate (**14**)[31], and adenosine (**15**)[32].

Compound **1** was obtained as colorless amorphous powder, and gave a positive Liebermann-Burchard test. It showed a quasi-molecular ion peak at *m/z* 1105.5461 $[M+H]^+$ in the positive HR-FAB-MS, corresponding to the molecular formula $C_{53}H_{85}O_{24}$. The NMR data agreed with those the literature[23]. Complete assignment of its structure was done by alkali and acidic hydrolysis in addition to 1D- and 2D-NMR experiments. Sugar sequencing was achieved by 1H-1H-COSY, 1D-total correlation spectroscopy (TOCSY), HMQC and HMBC. GC analysis of the trimethylsilyated sugars in the acid hydrolysate afforded D-glucose (t_R 9.30 min), L-rhamnose (t_R 7.28 min), D-fucose (t_R 7.50 min), and D-xylose (t_R 8.09 min). The structure of compound **1** was thus elucidated as 3-*O*-β-D-glucopyranosyl presenegenin-28-*O*-[β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl (1→2)-β-D-fucopyranosyl ester, i.e. polygalasaponin XXVIII [23].

The spectroscopic features indicated that **2** is closely related to **1** except for the presence of one more sugar unit in the sugar sequence at C-28. Comparison of the ^{1}H- and ^{13}C-NMR data of the sugar part of **2** and **1** and analyses of HMBC and NOESY correlations indicated that **2** contained a β-D glucosyl moiety linked to C-3 and an extra β-D-galatopyranosyl unit linked *via* (1→4) to β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-β-D-fucopyranosyl residue at C-28. Acidic hydrolysis with 2N aqueous CF_3COOH yielded presenegenin. The trimethylsilylated hydrolysate was analyzed by GC and compared with the retention time of the pre-trimethylsilylated standard sugars to afford the sugar moieties containing D-xylose, L-rhamnose, D-fucose, D-galactose and D-glucose[24].

The major components **1**, and **2**, sibiricoses A_6 (**12**), 3',6-disinapoylsucrose (**13**), sibiricaxanthone B (**6**), 7-*O*-methylmangiferin (**8**), adenosine (**15**), and tenuifoliside A (**9**) were tested *in vitro* for their inhibitory activity of the isotope labeled RTI-55[3β-(4-iodophenyl)tropane-2β-carboxylic acid methyl ester] binding to NET protein[27]. Compounds **1** and **2** exhibited 77% and 28% inhibition at 4.53 μM and 3.95 μM, respectively. The active fraction had 70% inhibiting activity at 10 μg/mL while the positive control, desipramine, had an IC_{50} of 0.93 nM.

Our previous study demonstrated two oligosaccharide derivatives, polygalatenosides A and B from the same fraction[15]. They also exerted the inhibitory activity of norepinephrine transporter with IC_{50} values of 30.0 and 6.04 μM, respectively. These results indicated that multicomponents of *P. tenuifolia* may act by its active components in blocking norepinephrine reuptake.

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