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New cyclic tetrapeptides from *Nonomuraea* sp. TA-0426 that inhibit glycine transporter type 1 (GlyT1)

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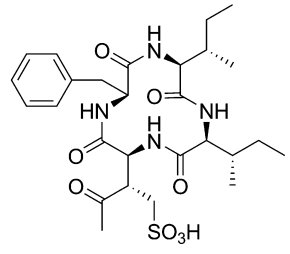
ABSTRACT

In the course of our search for natural antipsychotic agents, we isolated five new cyclic tetrapeptides from the fermentation broth of *Nonomuraea* sp. TA-0426. These compounds turned out to be analogues of WSS2220, which had been produced by the same actinomycete and showed strong inhibitory activity against GlyT1. Four of the present peptides exhibit more potent GlyT1 inhibitory activities than WSS2220.

Based on the glutamatergic hypothesis of schizophrenia, glycine transporter type 1 (GlyT1) is a promising target for antipsychotic agents.[1] Several GlyT1 inhibitors have been reported, and some of these agents are now being investigated in clinical trials.[1] We recently isolated a cyclic tetrapeptide, named WSS2220 (**1**), as a potent and selective inhibitor of GlyT1.[2] Compound **1** was produced by *Nonomuraea* sp. TA-0426 that was isolated from a soil sample collected at Henan Province, China. In our successive exploration of GlyT1 inhibitors, we were able to isolate five new tetrapeptides, 2–**6**, from a fermentation broth of TA-0426. In this paper, we report the isolation, structure determination and biological activity of these new peptides. The preliminary structure–activity relationship (SAR) based on the structures of the isolated compounds and their synthetic analogues is also described.

The *n*-BuOH extract (6.0 g) of TA-0426 was suspended in distilled water and extracted with EtOAc. The EtOAc extract was then separated by silica gel column chromatography (SCC), eluting with a $CHCl_3$–MeOH solvent system. The cyclic peptide-containing material, eluted with $CHCl_3$–MeOH 9:1, was further purified by reversed-phase HPLC (column; YMC-Pack ODS-AM 250 × 10 mm; id, 5 μm; YMC, flow rate; 2.5 mL/min), eluting with CH_3CN–H_2O 45:55, to give WSS2219 (**2**, 13.4 mg; rt = 15.6 min), WSS2221 (**3**, 3.7 mg, rt = 11.4 min), WSS2222 (**4**, 5.7 mg, rt = 12.0 min) and WSS2217 (**5**, 3.5 mg, rt = 9.7 min) as colorless powders. The $CHCl_3$–MeOH 19:1 eluent from SCC was purified by reversed-phase HPLC, eluting with CH_3CN–H_2O 65:35, to give WSS2218 (**6**, 15.2 mg, rt = 13.6 min) as a colorless powder (Fig. 1).



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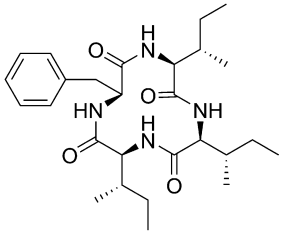
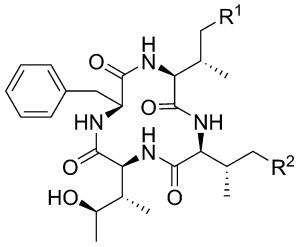


6



2 : R^1=CH_3, R^2=CH_3
3 : R^1=CH_3, R^2=H
4 : R^1=H, R^2=CH_3
5 : R^1=H, R^2=H

Figure 1. Structures of cyclic tetrapeptides **1–6**.

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The HRTOF-MS of **2** showed a quasi-molecular ion peak at *m/z* 525.3044 $[M+Na]^+$, corresponding to a quasi-molecular formula of $C_{27}H_{42}N_4O_5Na$ (Calcd for $C_{27}H_{42}N_4O_5Na$: 525.3053). IR absorptions at 1680 and 1522 cm^{-1} suggested the presence of amide functions. Based on an analysis of 1D and 2D NMR spectra, the presence of one phenylalanine (Phe) and two isoleucines (Ile) was easily deduced. The structure of the remaining amino acid residue was determined from 2D NMR spectral data (Fig. 2): the COSY spectrum indicated the connectivities H-2″/H-3″/H-6″ and H-4″/H-5″. The chemical shifts of C-4″ (δ 67.4) and H-4″ (δ 3.88) showed that C-4″ was an oxymethine carbon. The HMBC correlations between H-2″/C-1″ and H-6″/C-4″confirmed the connections of C-1″/C-2″ and C-3″/C-4″. Thus, this amino acid residue was confirmed to be 4-hydroxyisoleucine (4-OH Ile). The HMBC correlations H-2″/C-4″, H-5″/C-3″ and H-6″/C-2″ support the 4-OH Ile fragment.

We encountered a problem in determining the amino acid sequences; the four NH signals of **2** appear as an overlapped broad hump at δ 7.40–7.90 (in DMF-d_7),[3] which made it impossible to use them for sequencing by HMBC spectroscopy. Therefore, N-methylation of **2** was performed. Compound **2** was treated with CH_3I and NaH in DMF at 0 °C[4] to give pentamethylated compound **7** (30% after HPLC separation).[5] The strong *N*-Me signals of **7** showed intense HMBC cross-peaks and the following HMBC correlations clarified the amino acid sequences of **2**: NCH_3-2/C-2 and 1‴, NCH_3-2′/C-1 and 2′, NCH_3-2″/C-1′ and C-2″ and NCH_3-2‴/C-1″ and 2‴ (Fig. 2).

The configurations of Phe and Ile were determined to be L-Phe and L-Ile by hydrolysis (6 M HCl) followed by the advanced Marfey's method.[6] At the same time, the stereochemistry at the α-position of 4-OH Ile was also elucidated: HPLC of the L- and D-FDLA (1-fluoro-2,4-dinitrophenyl-5-leucineamide) derivatives of 4-OH Ile in the hydrolysates gave peaks at 6.69 and 10.48 min, respectively, which established that the α-position of 4-OH Ile had an *S*-configuration.[7] When the hydrolysis mixture was purified using a strong cation exchange (SCX) resin and SCC, cyclo-4-hydroxy isoleucine (**8**) was obtained in 18% yield (Scheme 1).[8] The coupling constants between H-2/H-3 and H-3/H-4 are 11.4 and 9.6 Hz, respectively, which indicates the anti/anti-configuration of H-2/H-3/H-4. Thus, the absolute stereochemistry of **8** was determined to be 2*S*,3*S*,4*R*. (2*S*,3*S*,4*R*)-Cyclo-4-hydroxyisoleucine was reported as a component of funebrine (**9**), which was isolated from the plant *Quararibea funebris*.[9] The coupling pattern of **8** was identical to the data in the literature.[9b] Thus, the structure of **2** was determined to be as shown in Figure 1. The structures of compounds **3–6** were determined essentially in the same manner as described for **2** and by comparison of their NMR data with those of **2**[10] (Table 1).

To investigate the structure–activity relationship, we synthesized H-Ile_3-Phe-OH (linear tetrapeptide, **10**), cyclo-(Ile_4-Phe) (cyclic pentapeptide, **13**) and cyclo-(Ile_5-Phe) (cyclic hexapeptide, **14**) (Scheme 2). Compounds **13** and **14** were prepared via cyclization of the linear peptides, **11** and **12**, respectively. The glycine-uptake

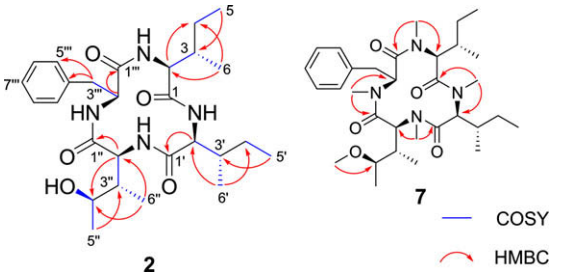
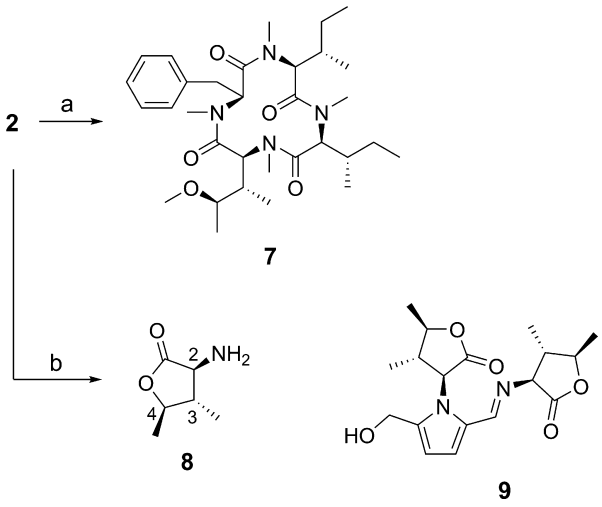


Figure 2. COSY and HMBC data for **2** and **7**.



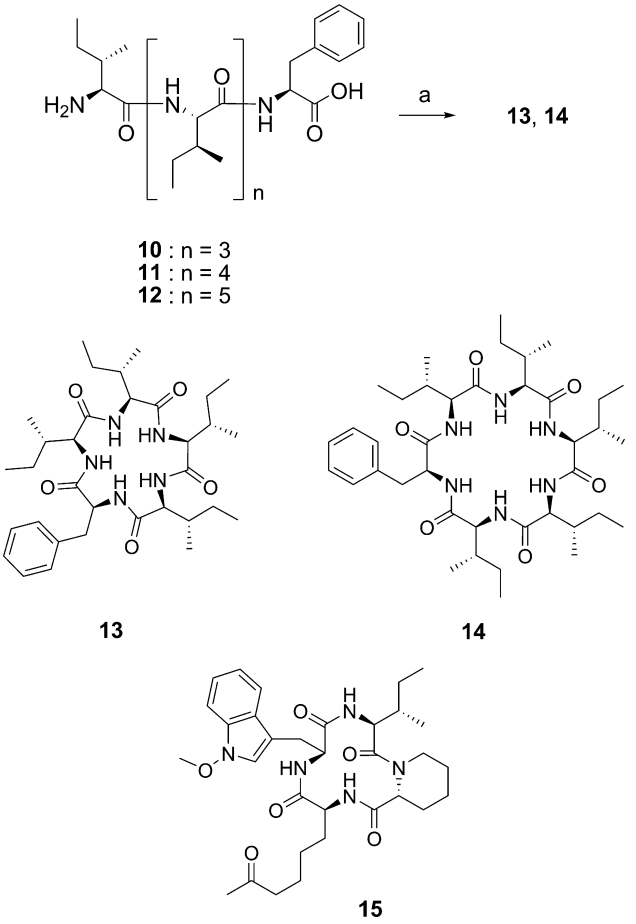
Scheme 1. Methylation and acid hydrolysis of **2**. Reagents and conditions: (a) CH_3I, NaH, DMF, 0 °C, 1 h; (b) 6 M HCl, 110 °C, 12 h. Structure of funebrine (**9**) is shown.

Table 1
^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) data for **2** in DMF-d_7.

Position		^{13}C	^1H
Ile-1	1	173.0	
	2	62.3	4.00 (dd, *J* = 11.0, 11.0)
	3	36.1	1.94 (m)
	4	26.5	1.20 (m), 1.60 (m)
	5	10.9	0.89 (t, *J* = 7.1 Hz)
	6	16.3	0.93 (t, *J* = 6.6 Hz)
	2-NH		7.40–7.90 (br)
Ile-2	1′	172.9	
	2′	62.0	4.02 (dd, *J* = 11.0, 11.0 Hz)
	3′	36.0	1.89 (m)
	4′	26.6	1.20 (m), 1.60 (m)
	5′	10.8	0.87 (d, *J* = 7.1 Hz)
	6′	16.2	0.91 (d, *J* = 6.6 Hz)
	2′-NH		7.40–7.90 (br)
4-OH Ile	1″	172.7	
	2″	60.3	4.09 (dd, *J* = 11.2, 9.6 Hz)
	3″	40.6	2.16 (m)
	4″	67.4	3.88 (m)
	5″	18.0	1.01 (d, *J* = 6.3 Hz)
	6″	11.1	0.73 (d, *J* = 6.6 Hz)
	2″-NH		7.40–7.90 (br)
Phe	1‴	173.1	
	2‴	58.3	4.57 (dt, *J* = 7.7, 8.8 Hz)
	3‴	37.7	3.14 (m)
	4‴	138.7	
	5‴, 9‴	130.1	7.20–7.35*
	6‴, 8‴	129.4	7.20–7.35*
	7‴	127.6	7.20–7.35*
	2‴-NH		7.40–7.90 (br)

* Not assigned precisely because of signal overlapping.

inhibitory activities and the GlyT1/GlyT2 selectivities of the isolated and synthesized peptides were measured (Table 2) as previously reported.[2a] T98G cells (2×10^4 cells/well) were plated in a 96-well plate and incubated at 37 °C in a humidified incubator. After 24 h, the cells were washed and incubated at room temperature with 250 nM [^3H]-glycine and test sample. After 15 min, the cells were washed and lysed with 0.5 M NaOH, a scintillation cocktail was added, and the cell lysates were counted using a scintillation counter.[11] The GlyT1/GlyT2 selectivities were also measured in the same way using COS7 cells transfected with rat GlyT1 or GlyT2. Of the tested samples, **2** showed the most potent inhibitory activity with high GlyT1 selectivity. The linear tetrapeptide (**10**)



Scheme 2. Synthesis of cyclic pentapeptide (**13**) and hexapeptide (**14**). Reagents and conditions: (a) HATU, HOAt, *i*-Pr_2NEt, DMF, 0 °C, 1 h. Structure of apicidin (**15**) is shown.

Table 2
GlyT inhibition activities (nM).

Compound	T98G	rGlyT1	rGlyT2
1	12.5	20.4	2260
2	3.5	3.0	278
3	5.5	10.2	574
4	5.0	11.4	450
5	4.0	11.1	542
6	21.2	29.6	226
10	16,400	N.T.	N.T.
13	655	N.T.	N.T.
14	1680	N.T.	N.T.
15	71.8	94.6	87.7
ALX5407	4.8	3.0	N.T.
ALX1393	N.T.	N.T.	21.1

ALX5407 (Tocris): selective inhibitor of GlyT1.
ALX1393 (Sigma–Aldrich): selective inhibitor of GlyT2.

showed weak glycine-uptake inhibitory activity in T98G. The cyclic pentapeptide (**13**) showed a moderate inhibitory activity, while cyclic hexapeptide (**14**) showed only weak activity. Apicidin (**15**),[12] a cyclic tetrapeptide that was reported as a histone deacetylase (HDAC) inhibitor, showed moderate inhibitory activity, but no selectivity was observed against GlyT subtypes. These results indicate that the cyclic tetrapeptide structure is important for strong inhibitory activity against GlyT, and GlyT1/GlyT2 selectivity is affected by the amino acid components.

We have isolated five novel cyclic tetrapeptides with selective GlyT1 inhibitory activities. A preliminary SAR study indicated that a cyclic tetrapeptide structure was necessary for strong inhibitory activity against GlyT, and the amino acid components affected the selectivity against GlyT subtypes.

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5. NMR data for **7**: ^{1}H NMR (500 MHz, pyridine-d_5): δ 0.24 (H-6″), 0.95 (H-5), 0.95 (H-5′), 1.03 (H-5″), 1.16 (H-6), 1.40 (H-6′), 2.15 (H-3), 2.71 (H-3″), 2.81 (2′-NCH_3), 3.07 (2-NCH_3), 3.18 (4″-OCH_3), 3.25 (2″-NCH_3), 3.39 (H-4″), 3.56 (2‴-NCH_3), 4.37 (H-2″), 4.62 (H-2), 5.30 (H-2′), 5.34 (H-2‴), 7.34 (H-5‴, H-9‴), 7.64 (H-6‴, H-8‴), 7.64 (H-7‴). ^{13}C NMR (125 MHz, pyridine-d_5): δ 9.4 (C-6″), 10.7 (C-5), 10.9 (C-5′), 15.8 (C-6′), 15.9 (C-5″), 16.9 (C-6), 24.3 (C-4′), 28.8 (C-4), 30.5 (2′-NCH_3), 30.5 (2″-NCH_3), 31.2 (2-NCH_3), 33.3 (2‴-NCH_3), 33.3 (C-3′), 33.5 (C-3), 37.0 (C-3‴), 37.7 (C-3″), 56.0 (C-2″), 56.3 (4″-OCH_3), 58.0 (C-2′), 59.5 (C-2‴), 70.3 (C-2), 76.1 (C-4″), 127.7 (C-4‴), 129.3 (C-6‴, C-8‴), 129.9 (C-5‴, C-8‴), 131.1 (C-7‴), 169.0 (C-1″), 169.5 (C-1′), 173.1 (C-1), 173.6 (C-1‴).
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8. NMR data for **8**: ^{1}H NMR (500 MHz, D_2O): δ 1.32 (3H, d, *J* = 6.4 Hz), 1.53 (3H, d, *J* = 6.0 Hz), 2.42 (1H, qdd, *J* = 6.4, 9.6, 11.4 Hz), 4.22 (1H, d, *J* = 11.4 Hz), 4.50 (1H, qd, *J* = 6.0, 9.6 Hz). ^{13}C NMR (125 MHz, D_2O): δ 173.2 (s), 82.2 (d), 55.8 (d), 42.9 (d), 17.3 (q), 12.3 (q).
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10. ^{13}C NMR data for **3** (125 MHz, DMSO-d_6): δ 9.8 (q; C-6″), 10.1 (q; C-6), 15.4 (q; C-5), 17.0 (q; C-5″), 19.1 (q; C-4′), 19.5 (q; C-5′), 25.0 (t; C-4), 29.0 (d; C-3′), 34.6 (d; C-3), 36.2 (t; C-3‴), 39.0 (d; C-3″), 56.5 (d; C-2‴), 58.4 (d; C-2″), 60.5 (d; C-2), 62.1 (d; C-2′), 65.4 (d; C-4″), 126.5 (d; Ar), 128.2 (d; Ar), 128.7 (d; Ar), 137.2 (s; Ar), 171.0 (s; C-1″), 171.3 (s; C-2′), 171.5 (s; C-1), 171.5 (s; C-1‴). ^{13}C NMR data for **4** (125 MHz, DMSO-d_6): δ 9.9 (q; C-6″), 10.0 (q; C-6′), 15.3 (q; C-5′), 17.1 (q; C-5″), 19.1 (q; C-4), 19.4 (q; C-5), 25.1 (t; C-4′), 29.0 (d; C-3), 34.7 (d; C-3′), 36.1 (t; C-3‴), 39.2 (d; C-3″), 56.4 (d; C-2‴), 58.3 (d; C-2″), 60.2 (d; C-2′), 62.3 (d; C-2), 65.5 (d; C-4″), 126.5 (d; Ar), 128.2 (d; Ar), 128.7 (d; Ar), 137.2 (s; Ar), 171.0 (s; C-1″), 171.3 (s; C-1′), 171.4 (s; C-1), 171.5 (s; C-1‴). ^{13}C NMR data for **5** (125 MHz, CD_3OD): δ 10.1 (q; C-6″), 16.6 (q; C-5″), 19.6 (q; C-4′), 19.6 (q; C-5′), 20.1 (q; C-4), 20.1 (q; C-5), 30.4 (d; C-3′), 30.5 (d; C-3), 37.3 (t; C-3‴), 40.5 (d; C-3″), 58.7 (d; C-2‴), 60.3 (d; C-2″), 64.3 (d; C-2′), 64.7 (d; C-2), 67.9 (d; C-4″), 127.8 (d, Ar), 129.5 (d; Ar), 130.2 (d; Ar), 138.3 (s, Ar), 174.5 (s; C-1″), 175.0 (s; C-1′), 175.2 (s; C-1), 175.3 (s; C-1‴). ^{13}C NMR data for **6** (125 MHz, CD_3OD): δ 10.4 (q; C-6″), 10.4 (q, C-6′), 10.6 (q; C-6), 15.4 (q; C-6″), 15.8 (q; C-6′), 15.8 (q; C-6), 26.8 (t; C-4″), 26.8 (t; C-4′), 26.9 (t; C-4), 36.0 (d; C-3″), 36.2 (d; C-3′), 36.3 (d; C-3), 37.4 (t; C-3‴), 58.5 (d; C-2‴), 62.3 (d; C-2′), 62.4 (d; C-2″), 62.7 (d; C-2), 127.8 (d; Ar), 129.4 (d; Ar), 130.1 (d; Ar), 138.4 (s; Ar), 175.1 (s; C-1″), 175.2 (s; C-1′), 175.3 (s; C-1‴), 175.5 (s; C-1).
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