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Synthesis of pseudopeptides based L-tryptophan as a potential antimicrobial agent

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Abstract—Four compounds named L-BTrpPA, L-Trp-*o*-PA, L-Trp-*m*-PA and L-Trp-*p*-PA, pseudopeptides constructed from pyridine and tryptophan units, were synthesized and tested against the Gram-positive, Gram-negative strains of bacteria and human pathogenic fungi. L-Trp-*o*-PA proved to be a broad-spectrum antimicrobial agent, showing a significant inhibition of the growth of Gram-positive bacteria (*Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Bacillus subtilis*, *Micrococcus luteus*), and pathogenic fungi (*Candida* spp., *Cryptococcus neoformans*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, *Aspergillus* spp., *Rhizopus nigricans*) tested and activity against Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa* and *Proteus vulgaris*, *Enterobacter aerogenes*) tested. The in vitro cell cytotoxicity of L-Trp-*o*-PA was evaluated using haemolytic assay, in which the compound was found to have low lytic property, even up to the concentration of 4000 μg/mL, it only lysed 6–7% of erythrocytes, which was 100-fold greater than the MICs (minimum inhibitory concentration).

Antimicrobial diseases are now more frequent than during the first half of the century, being still difficult to diagnose clinically. During the later half of the century, particularly during the past two decades, a number of different classes of antibacterial[1,2] and antifungal agents[3,4] have been discovered. At the present time, antibacterial sulfa drugs, nitrofuranes, penicillins, cephalosporins, tetracyclines, macrolides and oxaolidinones, and antifungal agents such as fluconazole, ketoconazole and miconazole, including amphotericin B, exhibit their antimicrobial activity.[1,4–6] Although there has been much progress in antibacterial and antifungal therapies, many problems remain to be solved for most antimicrobial drugs available. For example, although amphotericin B has strong antifungal activity, its serious nephrotoxicity often limits its clinical application.[7] Also many currently available azoles, such as fluconazole, ketoconazole and miconazole, lack antifungal activity against *Aspergillus fumigatus*, a common fatal pathogen in severe neutropenic patients. The use of azoles has also resulted in clinically resistant strains of *Candida* spp.[8] Moreover, appearance of multidrug resistant Gram-positive bacteria, in particular, methicillin-resistant *Staphylococcus aureus* is causing a serious problem. This situation highlights the need for screening new and more effective antibacterial and antifungal broad-spectrum drugs with low toxicity.

Indole-containing alkaloids have frequently been isolated from diverse marine invertebrates including bryozoans, coelenterates, sponges and tunicates.[9] Over the past few years, much attention has been paid to the search for specific indole secondary metabolites due to their novel structural features and broad spectrum of powerful biological activities.[10] The investigation of antimicrobial peptide alkaloids containing a tryptophan residue from a wide range of biological sources, and their synthetic derivatives is a novel inroad to new antifungal agents. Despite advances in biological therapies, difficulties with synthesis and isolation remain to be solved for the antimicrobial peptide alkaloids. Moreover, the use of simple tryptophan-containing compounds in medicine has barely been scratched. Furthermore, Blasi et al.[11] found that picolinic acid had protective activity against lethal infection of *Candida albicans* in mice. The effect of picolinic acid, a product of tryptophan catabolism, on anti-*Candida* activity of neutrophils was studied.[12] In this letter, we have

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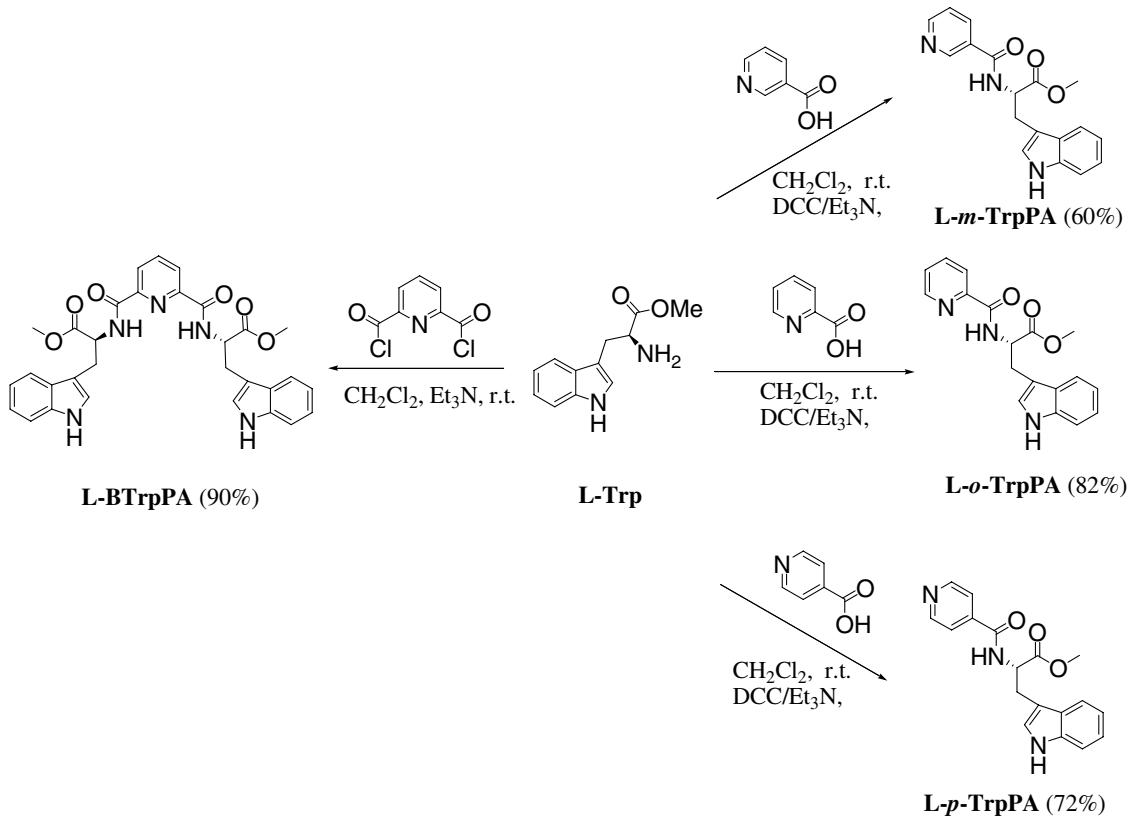
described the synthesis of four simple antimicrobial pseudopeptides containing pyridine and tryptophan units, named L-BTrpPA, L-Trp-*o*-PA, L-Trp-*m*-PA and L-Trp-*p*-PA, along with their in vitro antimicrobial activity and haemolytic assay.

The synthetic procedures for compounds L-BTrpPA, L-Trp-*o*-PA, L-Trp-*m*-PA and L-Trp-*p*-PA are illustrated in Scheme 1. L-BTrpPA was synthesized by condensation of 2,6-pyridinedicarbonyl dichloride, to form which 2,6-pyridine dicarboxylic acid undergoes reaction with $SOCl_2$, and L-tryptophan methyl ester in 90% yield at room temperature for 4 h. L-Trp-*o*-PA, L-Trp-*m*-PA, and L-Trp-*p*-PA were synthesized by condensation of the corresponding acid and L-tryptophan methyl ester by use of DCC/Et_3N coupling in 60–82% yield at room temperature for 12 h. All new compounds gave satisfactory IR, ^1H and ^{13}C NMR, ESI-MS and elemental data.[13]

The in vitro antibacterial activity was studied by the broth twofold dilution method[14] using pathogenic strains of *S. aureus*, methicillin-resistant *S. aureus* (three isolates), *Bacillus subtilis*, *Micrococcus luteus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Enterobacter aerogenes*. The experimental[15] result of antibacterial activity study indicated a variable degree of efficacy of L-BTrpPA and L-Trp-*o*-PA against different strains of bacteria (Table 1), measured as minimum inhibitory concentration (MIC, μg/mL) and minimum bactericidal concentration (MBC, μg/mL). The antibacterial activity was compared with those of standard antibacterial drugs, ampicillin, kanamycin and chloramphenicol.

A significant inhibitory activity superior to L-BTrpPA was displayed by L-Trp-*o*-PA against all pathogenic bacteria tested. L-Trp-*o*-PA exhibited a weak effect against Gram-negative bacteria (MIC 32–64 μg/mL), whereas none of the Gram-negative bacteria was sensitive to L-BTrpPA (MIC > 64 μg/mL). Noticeably, L-Trp-*o*-PA displayed a strong antibacterial activity against Gram-positive bacteria tested with MIC lower than 4 μg/mL, however, L-BTrpPA also displayed a weak activity against *S. aureus* (MIC 64 μg/mL) and *B. subtilis* (MIC 32 μg/mL), and none of any other Gram-negative bacteria was sensitive to the L-BTrpPA (MIC > 64 μg/mL). Interestingly, L-Trp-*o*-PA had good activities against methicillin-susceptible *S. aureus* and methicillin-resistant *S. aureus*, which were sensitive to the same extent (MICs range from 1 to 2 μg/mL). Actually, the MICs towards methicillin-resistant *S. aureus* (clinical isolates) were equal to the MICs of the standard drug chloramphenicol, while ampicillin and kanamycin were inactive up to the concentration of 64 μg/mL.

The in vitro antifungal activity was studied by the standard broth microdilution methods of the US National Committee for Clinical Laboratory Standards for yeasts[16] and filamentous fungi[17] using pathogenic strains of *C. albicans* (seven isolates), *Candida tropicalis* (two isolates), *Candida glabrata* (three isolates),



Scheme 1.

Table 1. MICs and MBCs of antibacterial compounds against pathogenic bacteria

Microorganisms	MIC[a] and MBC[b] (μg/mL)									
	L-BTrpPA		L-Trp-*o*-PA		Ampicillin		Kanamycin		Chloramphenicol	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-positive										
Staphylococcus aureus ATCC 25923[c]	64	—[g]	1	8	0.25	0.5	0.25	0.5	1	1
Staphylococcus aureus CMAH 0430[d]	NA[h]	—	1	4	>64[g]	—	>64	—	1	2
Staphylococcus aureus CMAH 0504[e]	NA	—	2	8	>64	—	>64	—	2	2
Staphylococcus aureus CMAH 0515[f]	NA	—	1	4	>64	—	>64	—	2	4
Bacillus subtilis ATCC 6633	32	>128	4	16	0.3	0.25	4	4	2	2
Micrococcus luteus CMCC(B) 28001	NA	—	4	8	0.3	0.5	0.25	0.25	2	4
Gram-negative										
Escherichia coli ATCC 25922	NA	—	64	—	32	64	0.25	0.25	2	4
Pseudomonas aeruginosa ATCC 27853	NA	—	32	>128	>64	—	64	>128	32	64
Proteus vulgaris CMCC(B) 49001	NA	—	32	>128	0.3	0.5	0.25	0.5	0.25	0.25
Enterobacter aerogenes CMCC(B) 45103	NA	—	64	—	>64	—	0.25	0.25	2	2

[a] MIC, minimal inhibitory concentration.
[b] MBC, minimal bactericidal concentration.
[c] Methicillin-susceptible *Staphylococcus aureus*.
[d] Methicillin-resistant *Staphylococcus aureus*, clinical isolates.
[e] Methicillin-resistant *Staphylococcus aureus*, clinical isolates.
[f] Methicillin-resistant *Staphylococcus aureus*, clinical isolates.
[g] — in the table means not tested when MICs are higher than 64 μg/mL.
[h] NA means no activity up to concentration of 128 μg/mL.

Table 2. MICs and MFCs of antifungal compounds against pathogenic yeasts

Microorganisms	MIC[a] and MFC[b] (μg/mL)						
	L-BTrpPA		L-Trp-*o*-PA		Amphotericin B		Fluconazole
	MIC	MFC	MIC	MFC	MIC	MFC	MIC
Candida albicans ATCC 10231	32	128	4	8	0.13	0.25	8
Candida albicans CMAH 0501[c]	32	128	4	16	0.25	1	16
Candida albicans CMAH 0503[d]	32	128	8	32	0.25	0.5	32
Candida albicans CMAH 0507[e]	64	—[n]	8	16	0.5	0.5	64
Candida albicans CMAH 0511[f]	32	128	2	4	1	2	16
Candida albicans CMAH 0520[g]	64	—	8	32	0.25	0.5	>64
Candida albicans CMAH 0527[h]	32	128	16	32	0.5	1	64
Candida tropicalis CMAH 0572[i]	NA	—	32	64	1	4	64
Candida tropicalis CMAM 0577[j]	NA	—	32	64	1	4	64
Candida glabrata CMAM 0592[k]	NA	—	4	16	2	2	32
Candida glabrata CMAM 0594[l]	NA	—	8	32	4	4	64
Candida glabrata CMAM 0597[m]	NA	—	8	32	2	4	>64
Cryptococcus neoformans ATCC 90112	NA	—	1	2	0.25	1	16
Saccharomyces cerevisiae SYM 3513	NA	—	4	8	0.25	0.5	4
Rhodotorula glutinis SYM 2209	32	128	1	2	16	>64	>64

NA means no activity up to concentration of 128 μg/mL.

[a] MIC, minimal inhibitory concentration.

[b] MFC, minimal fungicidal concentration.

[c] These fungi were clinical isolates, supplied by the First Clinical Hospital of China Medical University (Shenyang, China), among these *Candida albicans* CMAH 0520 and *Candida glabrata* CMAH 0597were both fluconazole-resistant *Candida* spp.

[d] These fungi were clinical isolates, supplied by the First Clinical Hospital of China Medical University (Shenyang, China), among these *Candida albicans* CMAH 0520 and *Candida glabrata* CMAH 0597were both fluconazole-resistant *Candida* spp.

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[j] These fungi were clinical isolates, supplied by the First Clinical Hospital of China Medical University (Shenyang, China), among these *Candida albicans* CMAH 0520 and *Candida glabrata* CMAH 0597were both fluconazole-resistant *Candida* spp.

[k] These fungi were clinical isolates, supplied by the First Clinical Hospital of China Medical University (Shenyang, China), among these *Candida albicans* CMAH 0520 and *Candida glabrata* CMAH 0597were both fluconazole-resistant *Candida* spp.

[l] These fungi were clinical isolates, supplied by the First Clinical Hospital of China Medical University (Shenyang, China), among these *Candida albicans* CMAH 0520 and *Candida glabrata* CMAH 0597were both fluconazole-resistant *Candida* spp.

[m] These fungi were clinical isolates, supplied by the First Clinical Hospital of China Medical University (Shenyang, China), among these *Candida albicans* CMAH 0520 and *Candida glabrata* CMAH 0597were both Fluconazole-resistant *Candida* spp.

[n] — in the table means not tested when MICs are higher than 64 μg/mL.

Table 3. Antifungal activity of L-Trp-*o*-PA and L-BTrpPA against Aspergillus spp. (14)[a]

Compound	MIC ± SD[b]	MIC_{50}[c]	MIC_{90}[d]	MIC range	MFC ± SD[e]	MFC_{50}[f]	MFC_{90}[g]	MFC range
L-BTrpPA	—	>128	>128	64–>128	ND[h]	ND	ND	ND
L-Trp-*o*-PA	33.60 ± 40.53	8	64	4–128	—	64	>128	16–>128
Amphotericin B	1.61 ± 1.16	1	4	0.5–4	2.96 ± 4.26	2	8	0.5–16
Fluconazole	>64	>64	>64	32–>64	ND	ND	ND	ND

[a] The total number of the tested pathogenic strains: *Aspergillus niger* (seven isolates), *Aspergillus fumigatus* (six isolates) and *Aspergillus flavus* (one isolate).

[b] MIC ± SD, Arithmetic mean of minimal inhibitory concentration values ± standard deviation.

[c] MIC_{50} and MIC $_{90}$, the MICs at which 50% and 90% of tested isolates are inhibited, respectively.

[d] MIC_{50} and MIC $_{90}$, the MICs at which 50% and 90% of tested isolates are inhibited, respectively.

[e] MFC ± SD, arithmetic mean of minimal fungicidal concentration values ± standard deviation.

[f] MFC_{50} and MFC_{90}, the MFCs at which 50% and 90% of tested isolates, are exhibiting 99% reduction of surviving, respectively.

[g] MFC_{50} and MFC_{90}, the MFCs at which 50% and 90% of tested isolates are exhibiting 99% reduction of surviving, respectively.

[h] ND, not determined.

Cryptococcus neoformans, *S. cerevisiae*, *R. glutinis*, *Aspergillus niger* (seven isolates), *Aspergillus fumigatus* (six isolates) and *Aspergillus flavus* (one isolate). The experimental[18] result of antifungal activity study indicated a variable degree of efficacy of L-BTrpPA and L-Trp-*o*-PA against different strains of fungi (Tables 2 and 3), measured as minimum inhibitory concentration (MIC, μg/mL) and minimum fungicidal concentration (MFC, μg/mL). The antifungal activity was compared with those of standard antifungal drugs, amphotericin B and fluconazole.

Results showed that L-Trp-*o*-PA had a wide and remarkable inhibitory effect against most of the tested strains including a broad range of yeasts and filamentous fungi. As shown in Table 2, L-Trp-*o*-PA displayed a significant antifungal activity against *C. albicans*, *C. glabrata*, *Cr. neoformans*, *Saccharomyces cerevisiae* and *R. glutinis* (MICs range from 1 to 16 μg/mL). In addition, a fungicidal activity was shown by L-Trp-*o*-PA, its MFC values (2–32 μg/mL) were identical to or just 2- or 4-fold higher than the corresponding MIC values. Noticeably, the antifungal activities of L-Trp-*o*-PA were superior or comparable to those of fluconazole against *Candida* spp., while slightly less potent than amphotericin B. The activities against current clinical fluconazole-resistant isolates were also measured. The activity of L-Trp-*o*-PA against *C. albicans* (CMAH 0520) and *C. glabrata* (CMAH 0597) was not changed, while the MICs of fluconazole were higher than 64 μg/mL. Interestingly, when tested against *R. glutinis*, amphotericin B showed minimum inhibitory concentration (MIC) about 16-fold greater than that of L-Trp-*o*-PA, and fluconazole was not active against this fungus (MIC > 64 μg/mL).[19] However, L-BTrpPA only exhibited selective and weak fungistatic activity just against *C. albicans* and *R. glutinis*, not against other yeasts.

As shown in Table 3, L-Trp-*o*-PA also had a moderate activity towards *Aspergillus* spp. The MIC_{50} value of L-Trp-*o*-PA at which 50% of the tested isolates were inhibited was 8 μg/mL. L-Trp-*o*-PA showed a better anti-*Aspergillus* activity than fluconazole and L-BTrpPA which were almost inactive up to the concentration of 64 μg/mL, but not comparable to the broad-spectrum antifungal drug Amphotericin B.

Moreover, L-Trp-*m*-PA and L-Trp-*p*-PA were tested against the Gram-positive, Gram-negative strains of bacteria and human pathogenic fungi. However, to our great surprise, the two compounds did not exhibit an activity against bacteria and fungi tested.

L-Trp-*o*-PA proved to be a broad-spectrum agent, showing a strong inhibition of the growth of all bacteria and fungi tested. We therefore determined the haemolytic assay in vitro of L-Trp-*o*-PA as a measure of its cell cytotoxicity.[20] Results demonstrated that concentrations up to 500 μg/mL of L-Trp-*o*-PA only lysed 2.52% of erythrocytes, even up to the concentration of 4000 μg/mL, it only lysed 6–7% of erythrocytes, which was 100-fold greater than the MICs. Compared with amphotericin B which lysed 50% of erythrocytes at the concentration of 30 μg/mL, about 30-fold higher than the arithmetic mean of MICs. It is clear that L-Trp-*o*-PA has low lytic properties. However, there was no significant difference in haemolytic activity between L-Trp-*o*-PA (6–7%) and chloramphenicol (14–15%) even up to 4000 μg/mL (Fig. 1).

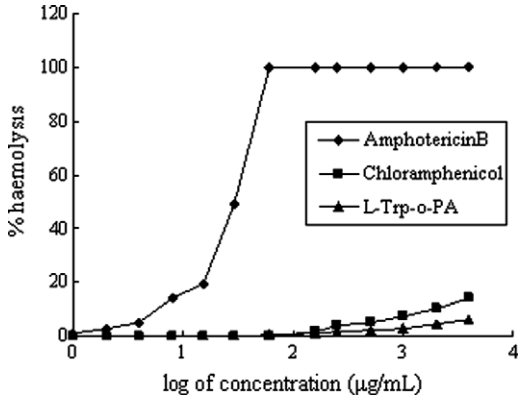

Figure 1. Haemolytic activity of L-Trp-*o*-PA.

On the basis of these results for activity of L-BTrpPA, L-Trp-*o*-PA, L-Trp-*m*-PA and L-Trp-*p*-PA, it appears that L-Trp-*o*-PA may be able to maintain a good antibacterial and antifungal activity against most of pathogenic bacteria and fungi and be an effective antimicrobial broad-spectrum drug. Especially, L-Trp-*o*-PA has a good activity against methicillin-susceptible and methicillin-resistant *S. aureus* and fluconazole-resistant *Candida* spp. from clinical isolates, which may be able to solve some problems of antimicrobial resistance. Thus, L-Trp-*o*-PA being less toxic than amphotericin B and chloramphenicol could be considered as a safer drug candidate and can be taken up for the development of suitable antimicrobial drug.

Acknowledgments

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13. Analytical data for L-BTrpPA: purification of the crude product by flash column chromatography on silica gel (CH_2Cl_2–ethyl acetate, 4:1) afforded the pure product L-BTrpPA. 90%. IR (KBr) ν (cm^{-1}): 3416, 3272, 3061, 3009, 2951, 1746, 1667, 1620, 1558, 1523, 1440, 1346, 1262. ^1H NMR (400 MHz) ($CDCl_3$) δ (ppm): 3.27–3.47 (4H, m), 3.85 (6H, s), 5.20–5.26 (2H, m), 6.65 (2H, d, $J = 2.4$ Hz), 6.95–7.00 (2H, m), 7.07–7.13 (4H, m), 7.49–7.52 (2H, t, $J = 6.0$ Hz), 8.02–8.10 (3H, m), 8.35–8.38 (4H, d, $J = 10.4$ Hz). ^{13}C NMR (100 MHz) ($CDCl_3$) δ (ppm): 173.2, 163.7, 148.8, 139.3, 126.2, 127.1, 125.8, 123.6, 122.4, 119.7, 118.3, 112.1, 108.8, 52.9, 52.1, 28.3. MS-ESI: *m/z*: positive polarity: 568.21 (M+1). Calcd for $C_{31}H_{29}N_5O_6$: C, 65.60; H, 5.15; N, 12.34%. Found: C, 65.58; H, 5.13; N, 12.30%. For L-Trp-*o*-PA: purification mixture was the crude product by flash column chromatography on silica gel (CH_2Cl_2–ethyl acetate, 4:1) afforded the pure product L-Trp-*o*-PA. 82%. IR (KBr) ν (cm^{-1}): 3412, 3286, 3066, 2931, 2863, 1702, 1681, 1649, 1545, 1436, 1373, 1279. ^1H NMR (400 MHz) ($CDCl_3$) δ (ppm): 3.35 (2H, d, $J = 5.4$ Hz), 3.60 (3H, s), 5.01–5.07 (1H, m), 6.94–7.18 (4H, m), 7.23 (1H, d, $J = 7.8$ Hz), 7.29–7.32 (1H, dd, $J = 2.1$, 5.4 Hz), 8.09 (1H, d, $J = 2.1$ Hz), 8.17 (1H, br s), 8.28 (1H, d, $J = 5.1$ Hz), 8.42 (1H, d, J = 8.1 Hz). ^{13}C NMR (100 MHz) ($CDCl_3$) δ (ppm): 172.3, 163.1, 150.8, 149.2, 146.1, 136.4, 127.6, 126.7, 123.2, 122.4, 119.7, 118.9, 111.5, 110.1, 53.4, 52.7, 28.2.. MS-ESI: *m/z*: positive polarity: 323.16 (M+1). Calcd for $C_{18}H_{17}N_3O_3$: C, 66.86; H, 5.30; N, 13.00%. Found: C, 66.81; H, 5.28; N, 12.95%. For L-Trp-*m*-PA: purification of the crude product by flash column chromatography on silica gel (ethyl acetate) afforded the pure product L-Trp-*m*-PA. 72%. IR (KBr) ν (cm^{-1}): 3385, 3280, 3058, 3016, 2943, 1732, 1664, 1618, 1567, 1531, 1438, 1340, 1259. ^1H NMR (400 MHz) ($CDCl_3$) δ (ppm): 3.37–3.47 (2H, m), 3.71 (3H, s), 5.08–5.13 (1H, q), 6.96 (1H, d, $J = 1.6$ Hz), 7.03–7.14 (3H, m), 7.22–7.29 (2H, m), 7.50 (1H, d, $J = 7.6$ Hz), 7.95 (1H, d, $J = 7.6$ Hz), 8.59 (1H, d, $J = 4$ Hz), 8.84 (2H, d, $J = 6.8$ Hz). ^{13}C NMR (100 MHz) ($CDCl_3$) δ (ppm): 172.4, 165.4, 152.1, 148.0, 136.4, 135.7, 129.9, 127.7, 123.7, 123.3, 122.5, 119.9, 118.6, 111.7, 109.8, 53.8, 52.8, 27.6. MS-ESI: *m/z*: positive polarity: 323.21 (M+1). Calcd for $C_{18}H_{17}N_3O_3$: C, 66.86; H, 5.30; N, 13.00%. Found: C, 66.83; H, 5.29; N, 12.97%. L-Trp-*p*-PA: purification of the crude product by flash column chromatography on silica gel (ethyl acetate) afforded the pure product L-Trp-*m*-PA. 60%. IR (KBr) ν (cm^{-1}): 3397, 3265, 3049, 3016, 2951, 1738, 1654, 1614, 1564, 1531, 1438, 1342, 1257. ^1H NMR (400 MHz) ($CDCl_3$) δ (ppm): 3.37–3.48 (2H, m), 3.72 (3H, s), 5.08–5.12 (1H, q), 6.96 (2H, d, $J = 2$ Hz), 7.02–7.16 (2H, m), 7.30 (1H, d, $J = 8.4$ Hz), 7.43 (2H, d, $J = 6$ Hz), 7.49 (1H, d, $J = 8$ Hz), 8.58 (2H, d, $J = 4.4$ Hz), 8.86 (1H, s). ^{13}C NMR (100 MHz) ($CDCl_3$) δ (ppm): 172.2, 165.3, 150.6, 141.2, 136.4, 127.8, 123.2, 122.5, 121.2, 119.9, 118.6, 111.7, 109.7, 54.0, 52.8, 27.6. MS-ESI: *m/z*: positive polarity: 323.17 (M+1). Calcd for $C_{18}H_{17}N_3O_3$: C, 66.86; H, 5.30; N, 13.00%. Found: C, 66.84; H, 5.27; N, 13.03%.
14. Jorgensen, J. H. In *Manual of Clinical Microbiology*; Murray, P. R., Baro, E. J., Pfaller, M. A., Tonover, F. C., Yolken, R. H., Eds.; American Society of Microbiology: Washington, 1995; pp 1275–1414.
15. In vitro antibacterial activity: Muller–Hinton broth (Difco) was employed as bacterial growth medium. Stock solutions of tested compounds were prepared in dimethylsulfoxide (Sigma D8418) and diluted in graded concentrations in the range of 0.25–128 μg/mL. Inocula-containing 10^5 cells/mL were obtained from broth cultures in log phase growth. The serial diluted chemical compounds' solutions inoculated with each bacterium were incubated on a rotary shaker at 35 °C for 24 h. The minimum inhibitory concentrations (MIC) of each tested compound were defined as the lowest concentration exhibiting no visible growth compared with the drug-free control wells. To measure the minimal bactericidal concentration (MBC), 100 μL of cell suspension was taken from each well that remained clear, subcultured on a Muller–Hinton agar plate and incubated at 35 °C for 24 h. The MBC was defined as the lowest concentration of the complex at which no growth occurred. A set of assay tubes containing only inoculated medium was kept as negative control and likewise solvent controls were also done simultaneously. All assays were performed in triplicate.
16. Reference method for broth dilution susceptibility testing of yeasts: approved standard. NCCLS document M27A. National Committee for Clinical Laboratory Standards, Wayne, PA, 1997.
17. Reference method for broth dilution susceptibility testing of filamentous fungi: approved standard. NCCLS document M38-A. National Committee for Clinical Laboratory Standards, Wayne, PA, 2002.
18. In vitro antifungal activity: RPMI 1640 (Gibco 31800022) which had been buffered to pH 7.0 with 0.165 M morpholinopropanesulfonic acid (Sigma) was used as the assay medium. Freshly grown fungi on slopes of sabouraud dextrose agar (logarithmic phase) were suspended with physiological saline, and the cell concentration was adjusted to a concentration of 10^4 cells/mL. The test compounds were initially dissolved in dimethylsulfoxide (DMSO) at a concentration of 6.4 mg/mL and further diluted with sterile growth medium. The final concentrations of the compounds ranged from 0.13 to 64 μg/mL. One hundred microliter of compounds' solutions was added to each well of a 96-well plate (Costar 3599). After incubation (100 μL/well, 5×10^3 cells/mL), the 96-well plates were incubated at 30 °C for 48 or 72 h, and the minimum inhibitory concentrations (MIC) of each tested complex were defined as the lowest concentration exhibiting no visible growth compared with the control wells. To measure the minimal fungicidal concentration (MFC), 100 μL of cell suspension was taken from each well that remained clear, subcultured on a sabouraud dextrose agar

plates and incubated at 30 °C for 48 h. The MFC was defined as the lowest concentration of the compound at which no growth occurred. A set of wells containing only inoculated medium was kept as negative control and likewise solvent controls were also done simultaneously. All assays were performed in triplicate.

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20. Haemolytic assay: human blood from healthy volunteers was collected in 10 mL Vacutainer tubes containing sodium heparin as anticoagulant. The cells were washed three times with calcium- and magnesium-free phosphate-buffered saline (PBS) and centrifuged at 2000*g* for 10 min. The third supernatant liquid was clear and colourless. Then 0.1 mL erythrocyte suspension diluted with PBS (erythrocyte concentration around 1.0×10^9 cells/mL) was mixed with 0.1 mL of test substances at a series of concentrations (1–4000 μg/mL). The mixtures were incubated at 37 °C for 1 h. After incubation, tubes were centrifuged at 2000*g* for 10 min. The supernatantes were transferred into 96-well polystyrene plates (Costar 3590, incorporated) and the optical density was measured at 540 nm using MTP120 microplate reader (Colona Electric, Japan). The values for 0% and 100% lysis were determined by incubating erythrocytes with PBS, and 0.1% (v/v) Triton X-100 (Amresco 0694), respectively. Assays were carried out in triplicate and the results were confirmed in three independent experiments.