Identification of anti-Parkinson’s Disease Lead Compounds from Aspergillus ochraceus Targeting Adenosin Receptors A_{2A}

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Two novel alkaloids compounds together with fifteen know metabolites were identified from Aspergillus ochraceus. The stereochemistry features of the new molecules were determined via HRESIMS, NMR, ECD, and XRD analyses. Amongst these, compounds two compounds exhibited potential efficacy as anti-Parkinson’s disease with the EC_{50} values of 2.30 and 2.45 μM, respectively. ADMET prediction showed that these compounds owned favorable drug-like characteristics and safe toxicity scores towards CNS drugs. Virtual screening analyses manifested that the compounds exhibited not only robust and reliable interactions to adenosine receptors A_{2A}, but also higher binding selectivity to A_{3A} receptors than to A_1 and A_2 receptors. Molecular dynamics simulation demonstrated the reliability of molecular docking results and the stability of the complexes obtained with the novel compounds and A_{2A} receptors in natural environments. It is the first time that anti-PD lead compounds have been identified from Aspergillus ochraceus and targeting adenosine A_{2A} receptors.

1. Introduction

Parkinson’s disease (PD) is a typical neurodegenerative disease. The main pathological characteristics of PD are the selective loss of dopaminergic neurons (DA) in the substantia nigra and the chronic accumulation of α-synaptic nuclear proteins forming Lewy bodies.[1] The clinical medications comprise mainly dopaminergic agents and non-dopaminergic drugs. However, the former, such as levodopa, carbidopa, rasagiline, etc., lacked long-term efficacy as well as being limited by adverse effects of the motor syndrome like “wearing-off”, oscillation, and dyskinesia.[2] The emerging non-dopaminergic agents, adenosine receptor type 2 A (A_{2A}) antagonists, via the blockade of A_{2A}R in striatopallidal neurons, appeared to possess prospective effectiveness on the control of the neurodegenerative processes of PD.[3] Nevertheless, few drugs were approved as A_{2A} antagonists targeting A_{2A}R for PD treatment. Thus, sincere efforts to discover more lead molecules of A_{2A}R antagonists for PD administration are urgently needed.

Aspergillus ochraceus (Aspergillaceae), a filamentous fungus, has been extensively investigated in recent decades for its secondary metabolites and related bioactivities. A. ochraceus contains a plethora of diverse secondary metabolites such as ochratoxins,[4] sesquiterpenoids,[5] pyrones,[6] lactones,[7] steroids,[8] and alkaloids,[9] while most of these metabolites have presented promising biological effects of antinsectan,[10] antibacterial,[11] anti-inflammatory,[12] antiviruses,[13] antitumors,[14] and neuroprotective.[15] Herein, seventeen diverse metabolites including a new benzodiazepine alkaloid, namely circumdatin N (7), and a new oxazole derivative, namely ochraceasol A (17), were discovered from A. ochraceus (Figure 1). The stereochemistry characteristics of new molecules were determined via extensive spectral methods, TD-DFT quantum-chemical prediction on ECD, and X-ray diffraction analyses. Amongst these isolates, compounds 14 and 15 exhibited promising protected effectiveness towards MPP^{+}-induced SH-SYSY cells with the EC_{50} values of 2.30 and 2.45 μM, respectively. ADMET prediction by Discovery Studio 2020 (DS20) presented that 14 and 15 have good drug-like characteristics of fitting Lipinski’s rules, CNS drugs features, and safe...
scores of toxicities. Virtual screening studies via LibDock and CDOCKER modules in DS20 showed that 14 has a higher dock score and approaching interaction energy compared to istraedfylline, an approved A$_2$R antagonist in Japan. AutoDock 4.2.6 with MGLTools 1.5.6 (ADT) analyses manifested that 14 exhibited robust and reliable interaction with A$_2$R (PDB code, 3EML) along with the highest binding affinities of $-10.27$ kcal/mol, and the lowest inhibition constants of $29.61$ nM, respectively. ADT also predicted that 14 presented higher binding affinity to A$_2$R than to A$_1$R (PDB code, 5N2S) and A$_3$R (PDB code, 1OEA). Besides, Molecular dynamics simulation demonstrated the reliability of molecular docking results and the stability of 14-A$_2$R complex in natural environments. These results provided us the first insight into that 14 could serve as a promising lead compound of targeting A$_2$R for exploring anti-PD drugs.

A modified PDA medium contained CYP450 oxidase inhibitor 8-geranyloxypsoralen was utilized for A. ochraceus culture. An ethanol extract from the whole rice fermentation of A. ochraceus was experienced extensive chromatographic methods to afford one new benzodiazepine alkaloid circumdatin N (7), and one new oxazole derivative ochracesol A (17), together with fifteen known isolates (1–6 and 8–16). According to the analyses of the NMR data with those reported in the literatures, these known compounds were confirmed as di-(2-ethylhexyl) phthalate (1),[13] ergosta-4,6,8(14),22-tetraen-3-one (2),[14] gymnasterone D (3),[15] isoycathisterol (4),[16] herbarulide (5),[17] demethylincinsterol A2 (6),[18] (+)-circumdatin F (8),[19] circumdatin G (9),[19] perlyrine (10),[20] notoamide B (11),[21] versicola-mide B (12),[22] notoamide F (13),[23] notoamide I (14),[24] stephadacin A (15),[25] and notoamide C (16).[25]

2. Results and Discussion

2.1. Structural Elucidation of the Secondary Metabolites

Compound 7 (circumdatin N), as the thirteenth new benzodiazepine derivative,[26] was deduced the molecular formula C$_{27}$H$_{27}$N$_{2}$O$_{3}$ by the HRESIMS pseudo molecular ion peak at m/z 330.0839 [M + Na]$^+$ (calcd for C$_{27}$H$_{27}$N$_{2}$NaO$_{3}$ 330.0855), demanding thirteen degrees of unsaturation. The IR spectroscopic analysis implied the molecule with the characteristic groups for hydroxyl (3375 cm$^{-1}$), carbonyl (1680 cm$^{-1}$), and aromatic rings (1614 cm$^{-1}$ and 1537 cm$^{-1}$). Owing to the data of the $^1$H and $^{13}$C NMR (Table 1), together with the HSQC spectrum (see Figures S1–S6 in the Supporting information), this metabolite was characterized the presence of one methyl ($\delta$$_{H}$ 14.9; $\delta$$_{C}$ 1.50 d, $J$ = 6.5 Hz), one methine ($\delta$$_{C}$ 49.3; $\delta$$_{H}$ 4.21 p, $J$ = 12.5, 6.5 Hz), seven olefinic/unsaturated carbons with protons ($\delta$$_{C}$ 109.8, 124.6, 128.6, 128.7, 128.8, 129.0, and 130.6; $\delta$$_{H}$ 7.44 d, $J$ = 2.8 Hz; 7.31, dd, $J$ = 8.8, 2.8 Hz; 7.58, br s; 7.75, m; 7.76, dd, $J$ = 7.6, 1.5 Hz; 7.60, br s; 7.64, m), and eight proton-free olefinic/unsaturated carbons ($\delta$$_{C}$ 122.0, 131.4, 133.3, 138.5, 153.1, 157.7, 160.9, and 166.8). These characteristics were very similar to those of (+)-circumdatin F (8)[19] and circumdatin G,[26] whilst the only differentiation between 7 and 8 was the additional presence of one hydroxyl substituted at C-13 of the benzene ring ($\delta$$_{C}$ 157.7) in 7, which fit the HRESIMS data analyses. The HMBC spectrum correlations (Figure 2) from H-12 to C-10, H-14 to C-12/C-16, H-15 to C-11, H-5 to C-3, H-6 to C-8, H-19 to C-18/C-2, Me-20 to C-18/C-19, and from NH ($\delta$$_{H}$ 8.74) to C-3/C-19/C-20 as well as the $^1$H–H COSY spin systems of H-4/H-5/H-6/H-7 and H-14/H-15 confirmed the planar structure of 7 as a benzodiazepine.
derivative, which was potentially biosynthesized via anthranilic acids and one alanine amino acid[9h].

To tackle the stereoscopic configuration of C-19 in compound 7, the electric circular dichroism (ECD) Cotton effects (CEs) were calculated using the time-dependent density functional theory (TD-DFT) methods. Gaussian 16 program was executed at the M06-2X-SCRF/def2-TZVP//M06-2X-SCRF/6-311G(d,p) level to collect the ECD spectra of 19R-7 to confirm the absolute asymmetric center of compound 17 should contain an oxazole ring, which also made the molecule to satisfy the preceding mentioned indices of the hydrogen deficiencies. Aside from the HSQC correlation signals, the planar skeleton of 17 was further characterized by 2D correlations of the HMBC and 1H-1H COSY spectra (Figure 2). Besides the HMBC correlations from H-3 to C-1 and H-5 to C-1/C-3, the presence of 1H-1H COSY cross-peaks of H-2/H-3 and H-5/H-6, together with their coupling constants approved the motif of para-disubstituted benzene. The 1-hydroxyethyl group was supported by the HMBC correlation signal of Me-13/C-12 along with the 1H-1H COSY spin system of H-12/H-13, which was assigned at C-8 of the oxazole ring by the presence of the HMBC correlations from Me-13/C-12 to C-8. Finally, the pivotal HMBC signal of H-3/C-11, H-5/C-11, and H-10/C-4 revealed the location of the oxazole ring at C-4 of the aromatic ring.

The above methods for the calculated ECD spectrum were also performed to confirm the absolute asymmetric center of 17. The calculated ECD curve of 12R-17 presented a good match with the experimental one (Figure 3). Fortunately, yielding high-quality colorless crystals via recrystallization, 17 followed the single-crystal X-ray diffraction analysis by Cu Kα radiation to be verified as 12R-7, 19R-7, and 12R-7 stereochemistry (Figure 4, Flack parameter 0.06(6), CCDC 1961585).

2.2. Biological Activities

1-methyl-4-phenylpyridinium (MPP+), a parkinsonian neurotoxin metabolized after a systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has been utilized as

Table 1. 1H NMR (400 MHz) and 13C (100 MHz) Data for compounds 7 and 17 (δ in ppm, J in Hz).

| No. | 7δH | 7δC | 17δH | 17δC |
|-----|------|------|------|------|
| 1   | 156.9 | 6.84 m | 116.9 | 6.84 m |
| 2   | 166.8 | 6.84 m | 116.9 | 6.84 m |
| 3   | 131.4 | 7.52 m | 127.1 | 7.52 m |
| 4   | 128.8 | 120.7 | 128.8 | 120.7 |
| 5   | 128.7 | 7.55 m | 127.1 | 7.55 m |
| 6   | 130.6 | 116.9 | 130.6 | 116.9 |
| 7   | 129.0 | 153.7 | 129.0 | 153.7 |
| 8   | 133.3 | 166.4 | 133.3 | 166.4 |
| 9   | 160.9 | 120.1 | 160.9 | 120.1 |
| 10  | 7.21 s | 120.1 | 7.21 s | 120.1 |
| 11  | 122.0 | 153.7 | 122.0 | 153.7 |
| 12  | 109.8 | 64.3 | 109.8 | 64.3 |
| 13  | 157.7 | 21.5 | 157.7 | 21.5 |
| 14  | 124.6 | 21.5 | 124.6 | 21.5 |
| 15  | 128.6 | 21.5 | 128.6 | 21.5 |
| 16  | 138.5 | 21.5 | 138.5 | 21.5 |
| 17  | 153.1 | 21.5 | 153.1 | 21.5 |
| 18  | 49.3  | 21.5 | 49.3  | 21.5 |
| 19  | 14.9  | 21.5 | 14.9  | 21.5 |

[a] Record in DMSO-d6. [b] Record in CH3OH-d4.

Figure 2. Key 2D NMR correlations of compounds 7 and 17.

Figure 3. Experimental ECD spectra of 7 and 17, and calculated ECD spectra of 19R-7, 19S-7, and 12R-7.

Figure 4. X-ray ORTEP drawing of 7 and 17.
an inducer towards SH-SY5Y cells for the study of the neuroprotective agents in PD.[25] Herein, along with levodopa as a positive control, the MTT assay was carried out to evaluate the anti-PD efficacy of all isolates through the PD model in vitro of MPP⁺-induced SH-SY5Y cells, as previously described. Intriguingly, most alkaloids generally showed potential neuroprotective effects to the MPP⁺ insult SH-SY5Y cells whereas other classes of compounds did not (Table 2). Amongst these alkaloids, compounds 14 and 15 exhibited dose-dependent potency with the EC₅₀ values of 2.30 and 2.45 μM, respectively (Table 2 and Figure 5).

### 2.3. ADMET Prediction

All alkaloids were predicted for the absorption, distribution, metabolism, and excretion in the ADME Descriptors module of DS20, covering molecular weight (MW), octanol/water partition coefficient (AlogP), octanol/water distribution coefficient (logD), hydrogen bond donors (HBD), polar surface area (PSA), rotatable bonds, aqueous solubility level, blood-brain barrier level (BBB Level), and human intestinal absorption level (Absorption Level) properties (Table 3). The predicted ADME properties of compounds 14 and 15 featured drug-like characteristics as fitting Lipinski’s rule of five states[27] and possessing appropriate profiles for CNS medicines.[28] Furthermore, safe and toxicity features were also considered in this research, and 14 and 15 displayed no Ames mutagenicity, less developmental toxicity potential, and relative safe data on rodent carcinogenicity (Table 4). These predictions imply that 14 and 15 are preferable safety, and they can be regarded as probable leading compounds.

### 2.4. Receptor-Based Virtual Screening

Adenosine receptors (ARs), as the G protein-coupled receptors, have several subtypes such as A₁, A₂A, and A₉, and in which A₂A exits in the basal ganglia with high density and plays a pivotal role in the regulation of motor movements. It was reported that highly selective A₂A antagonists could enhance the locomotor activity, reinforce the effects of levodopa, and attenuate the progress of dyskinesia by blocking A₂A.[29] Molecules with core motifs such as piperezine, pyrimidine/pyridine, or oxazole structures have been utilized as A₂A antagonists for PD therapy exits in the basal ganglia with high density and play a pivotal role in the regulation of motor movements.[30] Considering the encouraging activity on MPP⁺-induced SH-SY5Y cells and the analogous motifs of most alkaloids, virtual screening studies via LibDock and CDOCKER modules in DS20 were performed to predict hit compounds. The results exhibited that 14 has a higher dock score and an approaching CDOCKER interaction energy compared to istradefylline, an approved A₂A antagonist in Japan (Table 5).

### Table 2. Anti-PD Activities of compounds 1–17 on SH-SY5Y cells and MPP⁺-induced SH-SY5Y cells (μM).

| Compounds | CC₅₀[a] | EC₅₀[b] | SI[c] (CC₅₀/EC₅₀) |
|-----------|--------|--------|-------------------|
| 1         | 152.70 | 80.21  | 1.90              |
| 2         | 82.15  | 35.71  | 2.30              |
| 3         | 99.54  | 38.75  | 2.57              |
| 4         | 67.57  | 46.18  | 1.46              |
| 5         | 75.21  | 37.67  | 2.00              |
| 6         | 70.17  | 49.53  | 1.42              |
| 7         | 80.65  | 10.77  | 7.47              |
| 8         | 56.61  | 5.44   | 10.41             |
| 9         | 57.35  | 7.39   | 7.76              |
| 10        | 45.59  | 9.97   | 4.57              |
| 11        | 39.21  | 5.31   | 7.38              |
| 12        | 49.55  | 6.01   | 8.24              |
| 13        | 30.45  | 2.98   | 10.22             |
| 14        | 81.93  | 2.30   | 35.62             |
| 15        | 83.68  | 2.45   | 34.16             |
| 16        | 46.37  | 7.39   | 6.27              |
| 17        | 103.05 | 17.84  | 5.77              |
| Levodopa  | 154.9  | 2.06   | 75.19             |

[a] 50% cytotoxic concentration value. [b] 50% effective concentration value. [c] Selectivity Index.

**Figure 5.** Effects on SH-SY5Y cells and MPP⁺ insult SH-SY5Y cells viabilities of compounds 14 and 15 were evaluated using Levodopa (LD) as a positive control in vitro. Both values of CC₅₀ and EC₅₀ were calculated as mean values with standard deviations (n = 3, P < 0.05).
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Table 3. Prediction for the adsorption, distribution, metabolism, and excretion properties of compounds 7–17.

| Compounds | MW[a] | AlogP(b) | logD[c] | HBD[d] | PSA[e] | Rotatable Bonds[f] | Solubility Level[g] | BBB Level[h] | Absorption Level[i] |
|-----------|-------|----------|---------|--------|--------|-------------------|-------------------|-------------|-------------------|
| 7         | 307.3 | 1.75     | 1.75    | 2      | 82     | 0                 | 3                 | 3           | 3                 |
| 8         | 291.3 | 1.99     | 1.99    | 1      | 61.77  | 0                 | 2                 | 3           | 3                 |
| 9         | 307.3 | 1.75     | 1.75    | 2      | 82     | 0                 | 3                 | 3           | 3                 |
| 10        | 264.3 | 2.99     | 3.21    | 2      | 62.05  | 2                 | 2                 | 3           | 2                 |
| 11        | 447.5 | 1.47     | 1.47    | 2      | 87.74  | 0                 | 3                 | 3           | 3                 |
| 12        | 447.5 | 1.47     | 1.47    | 2      | 87.74  | 0                 | 3                 | 3           | 3                 |
| 13        | 461.2 | 2.62     | 2.62    | 2      | 83.66  | 1                 | 2                 | 3           | 3                 |
| 14        | 445.5 | 2.72     | 2.72    | 2      | 91.5   | 0                 | 3                 | 3           | 3                 |
| 15        | 431.3 | 3.16     | 3.16    | 2      | 74.43  | 1                 | 1                 | 3           | 3                 |
| 16        | 449.5 | 2.11     | 2.11    | 2      | 87.74  | 2                 | 3                 | 3           | 3                 |
| 17        | 205.2 | 1.37     | 1.35    | 2      | 66.49  | 2                 | 3                 | 3           | 3                 |

Profiles of a CNS drug candidate: (a) Molecular weights: suggested limits, < 500 D; preferred range, < 450 D. (b) ACD calculated Logarithm of octanol/water partition coefficient: suggested limits, 2–5; preferred range, 2–4. (c) Logarithm of the octanol/water distribution coefficient (at a given pH 7.4): suggested limits, 2–5; preferred range, 2–4. (d) Hydrogen bond donors: suggested limits, 0–3; preferred range, 0–1. (e) Polar surface area: suggested limits, < 90; preferred range, < 70. (f) Suggested limits, 0–8. (g) Extremely low, 0; very low, 1; low, 2; good, 3. (h) Blood-brain barrier: very high penetrant, 0; high, 1; medium, 2; low, 3; undefined, 4. (i) Human intestinal absorption level: good, 0; moderate, 1; poor, 2; very poor, 3.

Table 4. Prediction for the toxicities of compounds 7–17.

| Compounds | Rat NTP[a] | Mouse NTP[b] | Ames mutagenicity[c] | DTP[d] |
|-----------|------------|--------------|----------------------|--------|
|           | Male       | Female       |                      |        |
| 7         | –3.22      | –1.92        | –2.29                | –3.61  |
| 8         | –0.933     | –0.0252      | –3.1                 | –1.5   |
| 9         | –1.8       | –0.175       | –2.76                | –4.34  |
| 10        | 2.41       | 1.11         | –0.497               | –0.217 |
| 11        | 0.38       | –8.28        | –3.79                | 2.01   |
| 12        | 0.38       | –8.28        | –3.79                | 2.03   |
| 13        | 0.583      | –7.4         | –4.83                | 2.07   |
| 14        | 0.708      | –7.34        | –4.99                | 2.29   |
| 15        | 1.5        | –8.77        | –3.39                | 2.02   |
| 16        | –0.523     | –11.3        | –6.31                | 2.34   |
| 17        | –0.752     | –1.13        | –1.68                | –1.36  |

[a] U.S. National Toxicology Program: noncarcinogen, NTP < 0.3; carcinogen > 0.8. (b) Nonmutagen, < 0.3; mutagen, > 0.8. (c) Developmental toxicity potential: nontoxic, DTP < 0.3; toxic, > 0.8.

Table 5. Details of LibDockScore, CDOCKER Interaction Energy (kcal/mol), binding energies (kcal/mol), and inhibition constant (K) of compounds 7–17.

| Compounds | LibDockScore | CDOCKER Interaction Energy | A(2R) (PDB code: 3EML) Binding Constant | A(2R) (PDB code: S N25) Binding Constant | A(2R) (PDB code: 1OEA) Binding Constant |
|-----------|--------------|---------------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| 7         | 104.66       | –40.15                    | –8.00                                  | 1.36 μM                                | 2.75 μM                                |
| 8         | 100.80       | –36.64                    | –7.79                                  | 1.94 μM                                | 2.02 μM                                |
| 9         | 107.02       | –40.82                    | –8.05                                  | 1.25 μM                                | 2.48 μM                                |
| 10        | 98.06        | –43.66                    | –7.71                                  | 2.21 μM                                | 3.94 μM                                |
| 11        | 115.27       | –42.37                    | –8.97                                  | 268.03 nM                              | 4.82 μM                                |
| 12        | 113.11       | –43.84                    | –8.92                                  | 287.03 nM                              | 1.42 μM                                |
| 13        | 116.75       | –45.85                    | –9.00                                  | 251.78 nM                              | 8.14 μM                                |
| 14        | 126.00       | –45.93                    | –10.27                                 | 29.61 nM                               | 7.16 μM                                |
| 15        | 117.97       | –42.88                    | –9.57                                  | 96.89 nM                               | 7.16 μM                                |
| 16        | 122.45       | –45.29                    | –7.36                                  | 40.40 μM                               | 1.46 μM                                |
| 17        | 79.32        | –31.47                    | –5.95                                  | 43.60 μM                               | 4.64 μM                                |
| Istradefylline | 121.74      | –51.76                    | –7.83                                  | 1.82 μM                                | 3.71 μM                                |

selective or nonsensitive binding affinities to A(2R) of 14 were screened via the same docking procedure. Computational screening results showed that 14 presented higher binding affinity and lower inhibition constant to A(2R) than respective to A(1R) (PDB code, 5N25) with –6.20 kcal/mol and 28.46 μM, and A(1R) (PDB code, 1OEA) with –7.23 kcal/mol and 5.02 μM (Table 5), which means a preferable selectivity of 14 for binding to A(2R).

2.5. Molecular Dynamics Simulations

To demonstrate the reliability of molecular docking results and the stability of A(2R)-A complex, the RMSD, and the RMSF of A(2R)-A were investigated via molecular dynamics simulation (Figure 6). The results exhibited that the RMSD of the complex tended to converge to equilibrium during 162 ps, which indicated the system began to reach a stable state. The average
RMSD of the complex was 0.26 nm, which suggested the result of molecular docking is reliable. The RMSF values in the process of molecular dynamics simulation were calculated to study the flexibility of the molecular docking complex. The higher the RMSF value of amino acid is, the more flexible it is in the process of binding. During the whole simulation process, the average RMSF of the complex was only 0.12 nm to the original complex 3EML, suggested that the conformational shift of the complex was small. Statistical residue analysis on non-bond interactions of 14-Aβ2R complex (see Table S1 in the Supporting information) showed that top 5 amino acid residues Phe168, Ile274, Leu167, Tyr271, Ile66, and Glu169 presented favorable interactions via hydrophobic and/or hydrogen-bond interactions, and only limited counts exhibited unfavorable interactions (Figures 7A and 7B); top 5 residues Glu169, Phe168, Tyr271, Leu167, and Ile66, as well as top 5 residues Ile274, Leu167, Phe168, Ile66, and Tyr271, displayed hydrogen-bond and hydrophobic interactions, respectively (Figures 7C and 7D).

2.6. Visual Graphic of 14-Aβ2R Complex Docking

Molecular docking results were analyzed via PyMOL Molecular Graphics System 2.4 and DS20, which presented the sizes and locations of active sites, hydrophobic or hydrophilic, and hydrogen-bond interactions, as well as bonding distances i.e., interaction radius less than 5 Å between the binding sites and the docked compound. Compounds were docked to the active pockets of proteins. Binding poses were observed, and the interactions between ligands and receptors were characterized. The best energetically favorable conformations were chosen from each compound.

The perspective of binding interactions of 14-Aβ2R was visualized by PyMOL and DS20 (Figure 8), which showed that the molecule can bind well to the active pocket of Aβ2R and interact with Phe168 and Glu169 via hydrogen bonds along with the distance of 3.0 Å and 3.7 Å, respectively, with Phe168 via π-π stacked, and form hydrophobic interactions with Leu167, His264, Leu267, Met270, Glu169, Met270, Leu249, Ile274, and Ile66.

3. Conclusions

In summary, seventeen diverse metabolites were discovered from the marine-derived fungus Aspergillus ochraceus MCCC 3A00521, including a new benzodiazepine alkaloid (7), and a new oxazole derivative (17). Compounds 14 and 15, as prenylated indole alkaloids with piperazine motifs, featured promising potency with their respective EC_{50} values of 2.30 and...
2.45 μM. Interestingly, compounds 11–13 and 16, which have similar skeletons to those of 14 and 15, also present lower EC_{50} values towards anti-PD than other types of compounds. ADMET prediction showed 11–16 possessed good drug-like characteristics, appropriate features for CNS drugs, and preferable safety scores of toxicities. Extensive virtual screening suggested that these prenylated indole alkaloids with piperazine motifs, 11–16, docked with 3EML, exhibited higher LibDock scores and considerable CDOCKER interaction energies compared to other alkaloids. It is noteworthy that compound 14 not only has better anti-PD activity in vitro, but also exhibited a higher Libdock score than the reference compound istradefylline better performance than 1

Figure 8. Visual graphic interactions of molecular docking between 14 and A_2A_R. Left: the whole drawing of 14-A_2A_R; Right: the amplified drawing of 14 docking in pocket sites of A_2A_R (The above: 3D graphic; The Below: 2D graphic).

Experimental Section

General Experimental Procedures

TLC was accomplished by silica gel 60 F_254, 305 (Qingdao Ocean Chemical Co., Ltd., China). Silica gel (80–120 and 200–300 mesh; Qingdao Ocean Chemical Co., Ltd., China), RP-C_18 silica gel (spherical, 20–45 μm, Santai Technologies, Inc., China), and Sephadex LH-20 (Beijing Solarbio Science & Technology Co., Ltd., China) were utilized for column chromatography. HPLC was completed on an Essentia Prep LC-16P with a UV detector via an RP-C18 column (5 μm, 10 × 250 mm, YMC-Pack, ODS-A), Thermo Fisher LC-LTQ-Orbitrap XL spectrometer was used to collect the HRESIMS data. Bruker AM-400 spectrometer was employed to obtain NMR data as well as taking the solvent peaks (CH_3OH-d_4: δ_4 3.1, δ_6 49.10; DMSO-d_6: δ_6 2.50, δ_6 3.95) as internal references of the 1H and 13C NMR chemical shifts. JASCO J-810 spectrometer was taken to carry out the ECD spectra measurement. Infrared spectra data were collected via Bruker Vertex 70 equipment. Ultraviolet spectra were analyzed by a Varian Cary 50 instrument. The single-crystal X-ray diffraction data were gathered by a Bruker APEX DUO diffractometer under the graphite-monochromated Cu Kα radiation. The optical rotation measurement was fulfilled by a Perkin-Elmer 341 polarimeter.

Strain Material

The voucher specimens of Aspergillus ochraceus MCCC 3A00521 were provided by the Marine Culture Collection of China, which was isolated from the Pacific Ocean. The inoculated strain has been preserved in the Strain Preservation Centre of the School of Life Sciences, Hubei University, China.

Fermentation and Isolation

A. ochraceus MCCC 3A00521 were cultured onto two different PDA culture plates (one only contained PDA, namely PDA-Control; another was added a kind of CYP450 oxidase inhibition 8-geranyloxyisoporenamino into PDA, namely PDA-Test), then inoculated at 28 °C for a week as seed plates. Two types of methanol extracts of A. ochraceus were subjected to LC-MS/MS system to obtain their MS spectra (Figure 9). Since more m/z peaks emerged in Figure 9 B, this culture strategy was adopted to amplify the cultivation of A. ochraceus. The seed plates were successively hatched into 50 L
conical flasks (100 × 500 mL, each consisting of the sterilized rice medium) at 25 °C for four weeks for a solid fermentation. The fermented cultures were immersed into 100% ethanol at room temperature for seven days, then a crude extract was obtained after removal of residual ethanol by vacuum distillation. Afterward, the crude extract was sequentially eluted with petroleum ether, chloroform, and ethyl acetate to afford three partitions, respectively. These three portions consecutively underwent column chromatography analyses of silica gel, MPLC (RP-C18 spherical), and Sephadex LH-20. Eventually, semi-preparative HPLC was performed to yield compounds 1–6 (from the section of petroleum ether), compounds 7–16 (from the section of chloroform), and compound 17 (from the section of ethyl acetate).

Circumdatin N (7): Colorless crystals, mp 168–170 °C; [a]D 25 +150.9 (c 0.35, CH3OH); ECD (MeOH) λ (α) 209 (−5.94), 226 (+3.37), 254 (−2.22), and 278 (+0.93) nm; UV (CH3OH) λmax (log ε) = 230 (4.56), 277 (4.06) nm; IR (KBr) νmax = 3375, 3064, 1680, 1661, 1615, 1486, 1469, and 1385 cm⁻¹; H and ¹³C NMR data, see Table I; HRESIMS [M + Na]⁺ m/z 330.0839 (calcd for C13H11NO6, 330.0855).

Ochracesol A (17): Colorless crystals, mp 135–137 °C; [a]D 25 +30.7 (c 0.61, CH3OH); ECD (MeOH) λ (α) 211 (+0.20), 229 (−0.11), 249 (+0.12), 271 (+0.18), and 284 (−0.09) nm; UV (CH3OH) λmax (log ε) = 274 (4.43), 208 (4.06) nm; IR (KBr) νmax = 3302, 3390, 2938, 1620, 1505, 1446, and 1384 cm⁻¹; H and ¹³C NMR data, see Table I; HRESIMS [M + H]⁺ m/z 206.0724 (calcd for C10H10NO6, 206.0817).

Single-Crystal X-Ray Data for Circumdatin N (7)
C13H11NO6, M = 205.21, monoclinic, a = 26.6757(3) Å, b = 4.44940(10) Å, c = 12.2082(10) Å, α = 90.00°, β = 98.7560(10)°, γ = 90.00°, V = 1432.11(4) Å³, T = 100.01(10) K, space group C2, Z = 4, μ(CuKα) = 0.829 mm⁻¹, 13305 reflections measured, 2840 independent reflections (Rint = 0.0255). The final R values were 0.0417 (I > 2σ(I)). The final wR2(F2) values were 0.1015 (I > 2σ(I)). The final R values were 0.0419 (all data). The final wR2(F2) values were 0.1017 (all data). The goodness of fit on F² was 1.136. Flack parameter = 0.06(6). The Hooft parameter was −0.00(2).

Single-Crystal X-Ray Data for Ochracesol A (17)
C10H10NO6, M = 206.08, monoclinic, a = 26.5766(3) Å, b = 6.8806(2) Å, c = 13.1934(9) Å, α = 90.00°, β = 119.0065(11)°, γ = 90.00°, V = 1043.78(16) Å³, T = 100.01(10) K, space group P21, Z = 4, μ(CuKα) = 0.798 mm⁻¹, 20222 reflections measured, 4189 independent reflections (Rint = 0.0186). The final R values were 0.0305 (I > 2σ(I)). The final wR2(F2) values were 0.0779 (I > 2σ(I)). The final R values were 0.0306 (all data). The final wR2(F2) values were 0.0781 (all data). The goodness of fit on F² was 1.078. Flack parameter = 0.02(4). The Hooft parameter was 0.01(2).

Deposition Numbers 1961585 (for 17) and 1961586 (for 7) contain the supplementary crystallographic data for this paper. These data are provided free of charge by the joint Cambridge Crystallographic Data Centre and Fachinformationszentrum Karlsruhe Access Structures service www.ccdc.cam.ac.uk/structures

Cell Culture and Cytotoxicity Assay
SH-SYSY cells were incubated in DMEM medium with fetal bovine serum (10%), penicillin G (80 U/mL), and streptomycin (100 U/mL) under a moist atmosphere with 5% CO₂ at 37 °C. Cells were dispensed into a 96-well culture plate with 1 × 10⁴ cells per well. As being grown to 70% confluence, the cells were treated with diluted gradients of all isolates for 24 hours. The medium was discarded before the MTT solution (200 µL, 0.5 mg/mL) was added to each well. After further cultivation for 4 hours, the medium was removed. DMSO (150 µL) was successively added to each well and then shaken to mix it well. The parallel assays were repeated three times. The optical density (OD) of each well was measured via an Envision 2104 multilabel reader (Perkin Elmer, USA). The 50% cytotoxic concentration values (CC50) were calculated using Graphpad Prism 8.

Bioactivities Assay of Compounds on MPP⁺ Induced SH-SYSY Cells SH-SYSY cells were cultured as the above process. Then cells in the logarithmic growth phase were inoculated into a 96-well plate (1 × 10⁴ cells/well) with 6 multiple wells in each group. The assay was set up three groups: normal control group, being added 200 µL DMEM medium; model group, being added 200 µL DMEM medium containing MPP⁺ with the concentration 1 mM; drug group, one hour before added MPP⁺, being added designated concentrations of compounds or the positive control. All groups were cultivated for 24 hours. The following operations were performed similarly to the above-mentioned. The 50% effective concentration values (EC50) were simulated via Graphpad Prism 8.

Virtual Screening Details
Structures of isolated alkaloids were established by ChemOffice 2018 program, and the geometries energies were optimized via Gaussian 16 at the B3LYP/6-311G(d,p) level. PDB crystal structures were downloaded from RCSB PDB database and PDB site records were selected as the binding sites to screen the prepared alkaloids for selected Aβ-R antagonists. Virtual screening was implemented via the LibDock programme of DS20. The minimization algorithm was performed using Smart Minimizer at CHARMM Minimization Forcefield level. All binding poses of compounds were sorted based on the LibDockScore.

CDocker module of DS20 is a high precision procedure, which is based on CHARMM force field, and regarded proteins as rigid and ligands as flexible for molecular docking. PDB crystal structures were imported and prepared via DS20 tools. The previously treated alkaloids were used to process docking, the binding site spheres were defined according to PDB site records. Finally, a plurality of different conformations of each ligand-protein complex was obtained, which were further analyzed based upon CDocker interaction energies.

The created .mol2 files of prepared alkaloids by Gaussian 16 were saved as .pdb via PyMOL 2.4. 3D coordinates of A.R (PDB code, 5N2S), Aβ-R (PDB code, 3EML), and Aβ-R (PDB code, 1OEA) were downloaded as .pdb format from RCSB protein database. Autodock demands ligands and receptor portrayals in .pdbqt format files, which belong to the modified protein data bank [31] format involving atom type definitions, atomic charges, and topological information (rotatable bonds) for ligands. The .pdbqt molecules were submitted to Autodock Tool 4.2 (ADT) to generate .pdbqt files after being checked charges and detected torsion numbers. Being removed water molecules and redundant atoms, the macromolecules (receptors) were added hydrogens, Gasteiger charges, and were assigned AD4 atom types to create .pdbqt files. AutoGrid was utilized to generate grid maps. The grid boxes were established according to the active pockets of the original protein crystal structures. Docking works were performed with the default parameters under the Lamarckian Genetic Algorithm. Positional Root Means Quatre Deviation (RMSD) tolerance of 2.0 Å was considered optimal and clustered together for the advantageous binding search.
Molecular Dynamics Simulation Details

According to the docking results of CDOCKER, the 14-AhR complex with the best pose was selected and processed for molecular dynamics simulation. The simulation was carried out with the natural pressure as well as nearly 300 K temperature during the whole experiment. The CHARMM force field was applied, then the complex solvation was subjected in an orthorhombic cell shape and an explicit periodic boundary model. The standard dynamics simulation of 14-AhR complex was analyzed for RMSD to conformations and RMSF of amino acid residues.

Statistical Analysis

Data of the experiments were shown as means ± SD and analyzed via the Student's t-test or one-way ANOVA. Statistic data were achieved by using GraphPad Prism 8.0. The value of P < 0.05 was of statistical significance.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: adenosine receptors · Aspergillus ochraceus · alkaloids · drug discovery · Parkinson’s disease · virtual screening

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