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SYNTHESIS, CRYSTAL STRUCTURE, AND ANTI-GASTRIC CANCER ACTIVITY OF ETHYL 3-(3-AMINO-4-(METHYLAMINO)-N-(PYRIDIN-2-YL) BENZAMIDO)PROPANOATE

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A new heterocyclic compound ethyl 3-(3-amino-4-(methylamino)-N-(pyridin-2-yl)benzamido)propanoate (**1**), designed using 4-(methylamino)-3-nitrobenzoic acid (**2**) as a starting material is successfully obtained *via* a multiple synthesis route and finally characterized by IR, ^{1}H NMR, and single crystal X-ray crystallography. In addition, the *in vitro* anti-cancer activity of newly synthesized complex **1** is emulated against three human gastric cancer cell lines SGC-790, MKN-4, and MKN45.

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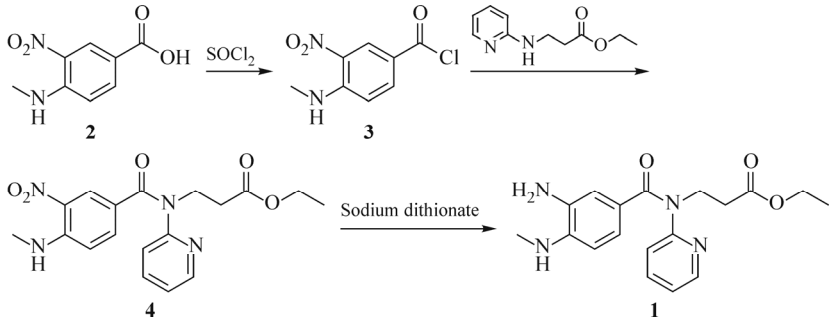
INTRODUCTION

Dabigatran etexilate is a thrombin inhibitor used to treat thromboses, cardiovascular diseases, etc. [1-3]. Ethyl 3-(3-amino-4-(methylamino)-N-(pyridin-2-yl)benzamido)propanoate (**1**) is one of the most important intermediates in the synthesis of Dabigatran etexilate [4-6]. Thus, much attention has been devoted to the synthesis, characterization, and crystal structure of compound **1** which has never been reported before [7-9].

The present work deals with the synthesis and characterization of title compound **1**. Compound **1** was synthesized by 4-(methylamino)-3-nitrobenzoic acid (**2**) as a starting material producing 4-(methylamino)-3-nitrobenzoyl chloride (**3**), then reacted with ethyl 3-(pyridin-2-ylamino)propanoate to obtain ethyl 3-(4-(methylamino)-3-nitro-N-(pyridin-2-yl)benzamido)propanoate (**4**), and title compound **1** was prepared after the reduction of compound **4** (Scheme 1). In addition,



Scheme 1. The synthesis route of compound **1**.

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the *in vitro* anticancer activity of compound **1** on three human gastric cancer cells (SGC-790, MKN-4, and MKN45) was further determined. Finally, molecular docking studies were utilized to further clarify its structure–activity relationship.

EXPERIMENTAL

Apparatus and materials. IR spectra (400-4000 cm^{-1}) were obtained using a Brucker Equinox-55 spectrophotometer. ^1H NMR spectra were obtained using a Varian Inova-400 spectrometer (at 400 MHz). Mass spectra were obtained using a micrOTOF-Q II mass spectrometer. The melting points were measured on a XT-4 micro melting apparatus, and the thermometer was uncorrected.

Synthesis and characterization of compounds 3, 4, and 1. 10 g of compound **2** was dissolved in 1 L of dichloromethane under the nitrogen atmosphere and cooled to 0-5 °C. Thionyl chloride was added to the reaction mixture for 1 h and the reaction mixture was heated to reflux for 5-6 h. Acid chloride compound **3** was obtained by removing excess thionyl chloride under vacuum and it was used at the next step.

Compound **3** (7.91 g, 0.0369 mol) was then dissolved in dichloromethane (100 mL) under an inert atmosphere and triethyl amine (7.47 g, 0.0738 mol) was added. To the reaction mixture a solution of ethyl-3-(pyridine-2-ylamino)propanoate (8.60 g, 0.0443 mol) in dichloromethane (20 mL) was added slowly at 0-5 °C. After the completion of the reaction, the reaction mass was diluted with water (150 mL) and the product was extracted with dichloromethane (200 mL). Compound **4** was obtained by distilling off the solvent under vacuum and purified by hexane. ^1H NMR (DMSO-d_6, 400 MHz): δ 1.11 (t, 3H, $–CH_2CH_3$), 2.65 (t, 2H, $–COCH_2$), 2.89 (d, 3H, $–NHCH_3$), 3.95 (q, 2H, $–NCH_2$), 4.17 (t, 2H, $–CO_2CH_2$), 6.82 (s, 1H, NH), 7.08 (d, 1H, Ar–H), 7.21 (m, 1H, Py–H), 7.30 (d, 1H, Py–H), 7.69 (s, 1H, Ar–H), 7.93 (s, 1H, Ar–H), 8.43 (m, 2H, Py–H). IR: ν_{max} (cm^{-1}): 3306 (s), 1632 (s), 1532 (s), 1406 (s), 1322 (m), 1187 (m), 1027 (s), 950 (w), 860 (w), 786 (w).

10 g of compound **4** was dissolved in 100 mL of a mixture of dioxane and water and heated to 50 °C. Sodium dithionate (24.90 g, 0.1208 mol) and potassium carbonate (1.11 g, 0.0081 mol) was added to the reaction mixture and maintained at 50 °C for 6 h. Title compound **1** was obtained by removing the solvent under vacuum and purified by recrystallization from ethylacetate. ^1H NMR (DMSO-d_6, 400 MHz): δ 1.11 (t, 3H, $–CH_2CH_3$), 2.65 (t, 2H, $–COCH_2$), 2.89 (d, 3H, $–NHCH_3$), 3.95 (q, 2H, $–NCH_2$), 4.17 (t, 2H, $–CO_2CH_2$), 4.53 (s, 2H, $–NH_2$), 5.06 (s, 1H, –NH), 6.67 (s, 1H, Ar–H), 6.75 (d, 1H, Ar–H), 7.10 (s, 1H, Ar–H), 7.56 (d, 1H, Py–H), 7.76 (t, 1H, Py–H), 8.38 (d, 1H, Py–H). IR: ν_{max} (cm^{-1}): 1607 (s), 1555 (s), 1397 (s), 1321 (m), 1193 (m), 1028 (w), 948 (w), 861 (w), 797 (w), 704 (w). HRMS (ESI^+), *m*/*z*: calc. for $C_{18}H_{22}N_4O_3$: 365.1590 [$M+Na^+$], found 365.1546.

Crystal structure determination. A suitable single crystal of compound **1** (obtained by slow volatilization of its CH_2Cl_2 solution) was carefully selected under an optical microscope and glued on thin glass fibers. The intensity data of **1** were collected on an Oxford Xcalibur E diffractometer. The empirical absorption corrections were applied to the data using the SADABS system. This structure was solved by a direct method and refined by the full-matrix least-squares method on F^2 using the SHELXS-97 program [10, 11]. All non-hydrogen atoms of **1** were refined anisotropically, and all the hydrogen atoms attached to carbon atoms were fixed at their ideal positions. Crystal data for $C_{18}H_{22}N_4O_3$ (M = 342.39 g/mol): triclinic, space group P-1 (No. 2), a = 8.3648(16) Å, b = 10.4702(12) Å, c = 10.9187(15) Å, α = 102.949(11)°, β = 98.162(13)°, γ = 97.403(13)°, V = 909.6(2) $Å^3$, Z = 2, T = 296.15 K, μ_{MoK_α} = 0.087 mm^{-1}, D_{calc} = 1.250 g/cm^3, 5914 reflections measured ($4.048 \le 2\theta \le 50.684°$), 3318 unique ($R_{int}$ = 0.0290, R_σ = 0.0484) which were used in all calculations. The final R_1 was 0.0624 ($I > 2\sigma(I)$) and wR_2 was 0.2013 (all data). CCDC number: 1867275.

Antitumor activity. Three human gastric cancer cells (SGC-790, MKN-4, and MKN45) were determined using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide] assay [12]. In this study, the cells were plated on 96 wells at $5 \cdot 10^3$. After attachment (24 h), the cells reaching 70-80% confluency were treated for 48 h with each compound at different concentrations or 1% dimethyl sulfoxide (DMSO) as a negative control. After 48 h incubation, 20 μL of the MTT solution (5 mg/mL in PBS) was added and incubated for additional 4 h. Subsequently, the medium was aspirated carefully,

and 150 μL of DMSO was added. After incubation for 15 min, the optical density was measured at 490 nm using a FlexStation 3 benchtop multi-mode microplate reader (Molecular Devices, USA). This assay measures the amount of formazan produced from MTT by dehydrogenase enzymes of metabolically active cells. Thus, the quantity of formazan produced is directly proportional to the number of living cells. Absorbance values of the treated cells were compared with the absorbance values of untreated cells. The IC_{50} value and IC_{90} were determined from non-linear regression equation. The results are presented as the average percentage viability to the negative control (1% DMSO).

Simulation details. The structure of compound **1** was optimized by density functional theory (DFT) using a B3LYP/6-31G(*d*) basis set. The structure optimization and energy calculations were performed with the GAUSSIAN 09 program.

AutoDock Vina v1.2 has been utilized to study the binding mode of compound **1** with tubulin [13]. Tubulin was used as downloaded without a further modification from the protein data bank (PDB ID: 1AS0). The geometric structures of compounds A and B were optimized through quantum chemistry calculations by Gaussian 09 at the B3LYP level of theory along with the 6-31G* basis set. AutoDockTools v1.5.6 has been adopted to transfer 1AS0 and the optimized structures of A and B to AutoDock Vina input files. Only polar hydrogen atoms are considered in these structures. The center coordinates of a search grid of tubulin were set to 38.15, –26.99 and 6.18, respectively, the length of the search grid was 15. All parameters needed for AutoDock Vina are used as default if not mentioned specifically. The calculated results were analyzed and visualized by PyMoL v1.8.6.

RESULTS AND DISCUSSION

Molecular structure. The structure of compound **1** ($C_{18}H_{22}N_4O_3$) was tested by the single crystal X-ray diffraction analysis. Its molecular structural unit is shown in Fig. 1. The C_5H_4N, $C_8H_9N_2O$, and $C_5H_9O_2$ groups are connected to the same N [(N(2)] atom. Within the structural unit, one NH_2 group and one CH_4N group are linked to the same phenyl group [C(7)–C(12)]. The C–C, C–N, and C–O bond lengths are in the range 1.363(4)-1.508(4) Å, 1.329(3)-1.468(3) Å, and 1.195(4)-1.445(4) Å, respectively. These bond distances fall in their normal ranges and they are comparable with the previously reported structures.

In addition, two kinds of classic hydrogen bonds (N–H…N and N–H…O) along with two kinds of non-conventional hydrogen bonds (C–H…N and C–H…O) could be observed in the packing structure along the *a* axis [14, 15]. The H14A–N1 and H14B–O1 distances are 2.44 Å and 2.36 Å with the D…A values of 2.811(3) Å and 2.703(3) Å, which fall in the normal ranges for the published hydrogen bond parameters (C–H…N and C–H…O) [16, 17]. The detailed information of hydrogen bonding is listed in Table 1. These multiple hydrogen bonds may play significant roles in its structural stability.

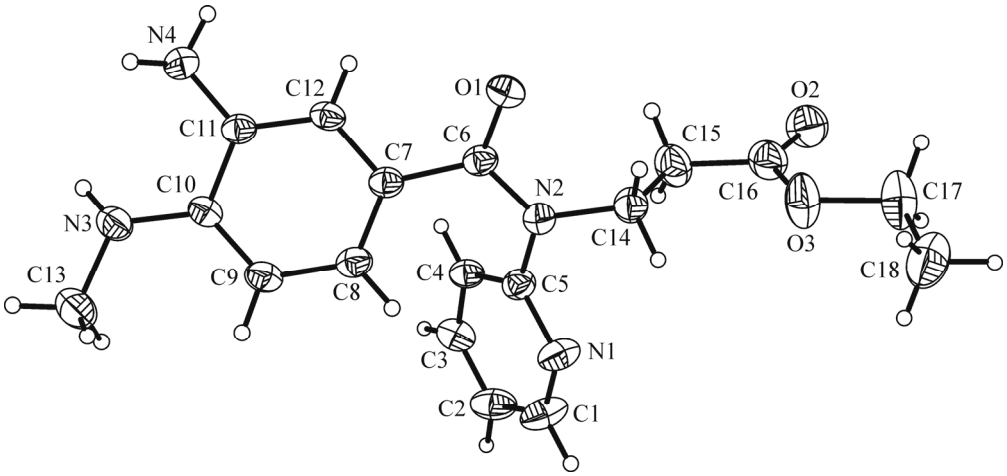

Fig. 1. Molecular structural unit of **1**.

TABLE 1. Hydrogen Bonds for Compound **1**

D–H…A	D–H	H…A	D…A	D–H…A
N3–H3…N4	0.87	2.48	2.773(3)	101
N3–H3…N4	0.87	2.43	3.205(3)	149
N4–H4A…O2	0.88	2.14	3.015(3)	170
N4–H4B…O1	0.88	2.23	3.072(3)	161
C14–H14A…N1	0.97	2.44	2.811(3)	102
C14–H14B…O1	0.97	2.36	2.703(3)	100

Symmetry code: 2–*x*, 1–*y*, 2–*z*; 1+*x*, *y*, 1+*z*; 2–*x*, 1–*y*, 1–*z*.

Anticancer activity. The interesting structural features of **1** encouraged us to test its cytotoxicity against a panel of human gastric cancer cell lines (SGC-790, MKN-4, and MKN45) along with normal mouse embryonic fibroblast (NIH 3T3) cells by the MTT assay method. The compounds were dissolved in DMSO and blank samples containing the same volume of DMSO were taken as controls to identify the solvent activity in this cytotoxicity experiment. Cisplatin was used as a positive control to assess the cytotoxicity of the test compounds. The results were analyzed by means of cell inhibition expressed as IC_{50} values and are shown Table 2. The IC_{50} values of complex **1** showed that it exhibited a significant activity against SGC-790, MKN-4, and MKN45 cell lines, which was almost equal to the activity of the well-known anticancer drug–cisplatin. The results of the *in vitro* cytotoxic activity studies have also indicated that the IC_{50} value of **1** against NIH 3T3 (normal cells) is found to be above 350 μM, which confirmed that the complex was very specific for cancer cells and even less toxic compared to cisplatin (IC_{50} = 198 μM).

TABLE 2. Cytotoxic Activity of Compound **1** and Cisplatin

Compound	Half maximum inhibitory concentration in μM (IC_{50})			
	SGC-790	MKN-4	MKN45	NIH 3T3
1	13.1±1.9	11.7±2.1	14.6±2.7	359±7
Cisplatin	12.5±0.7	13.8±0.8	10.5±1.8	198±11

TABLE 3. Experimental and Calculated Structural Parameters of the Selected Bond Lengths, Bond and Torsion Angles of Compound **1**

Parameter	X-ray	Calculation
Bond length, Å		
N1–C5	1.329	1.338
N2–C6	1.374	1.418
N3–C10	1.382	1.392
N4–C11	1.408	1.416
C15–C16	1.493	1.515
O3–C16	1.337	1.353
Angle / Torsions, deg		
N3–C10–C11	118.3	117.2
O1–C6–N2	119.5	119.9
N2–C14–C15	112.4	112.1
N3–C10–C11–N4	–2.7	–1.7
O1–C6–N2–C5	–163.4	–151.7
C14–C15–C16–O3	6.6	–43.1

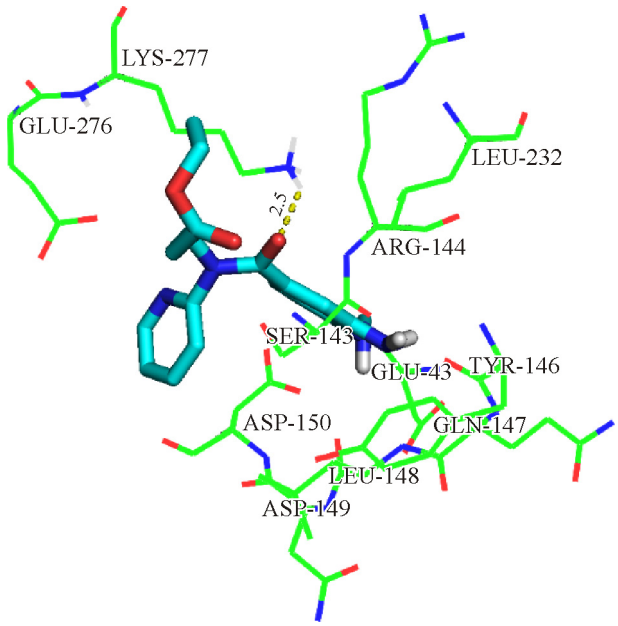

Fig. 2. The most favorable binding mode between compound **1** (stick) and the neighboring residues from protein 1AS0, the labels of the residues, the hydrogen bond as well as its length are shown explicitly.

Molecular docking. The autodock calculation was performed to investigate the polar interaction of compound **1** with respect to proteins, which was an important indicator to see the potential antivirus capability. Before the autodock calculation was implemented, the structure of compound **1** was optimized by the DFT method at the B3LYP/6-31G* level of theory. The initial structure for the optimization was taken from the experimental XRD result. The comparison of characteristic parameters is summarized in Table 3, from which we can conclude that the DFT calculation could precisely predict the bond and angle parameters. Slight discrepancies for torsions are observed because in the experiment, each single molecule in the crystal is in a relatively constrained environment, while no constrains are considered in the DFT calculation.

After the optimization, the possible interactions between compound **1** and protein 1AS0 were studied by autodocking. Eight possible binding modes were found, from which the most favorable binding mode was shown in Fig. 2. The corresponding affinity energy and the polar interaction length are –7.4 kcal/mol and 2.5 Å. From Fig. 2 we can see that not all heteroatoms are involved in polar interactions; only the carbonyl oxygen atom that is the linker of two ring structures has a polar interaction with residue LYS-277. The other functional groups such as ester, amino and imino groups do not have any interactions with neighboring residues ARG-144, SER-143, GLU-43, and ASP-150. From the above calculations we can see that compound **1** has a potential antivirus capability, which is in good agreement with the experimental observation.

CONCLUSIONS

In conclusion, we synthesized a novel heterocycle derivatives and characterized them *via* IR, ^{1}H NMR, HRMS, and single crystal X-ray diffraction. The MTT assay shows that complex **1** may act as novel anticancer drug in the future for its *in vitro* anticancer activities against three human gastric cancer lines SGC-790, MKN-4, and MKN45.

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AUTHOR CONTRIBUTIONS

L.-Z. Liu and K.-Y. Peng contributed equally to this work.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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