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RESEARCH ARTICLE

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Synthesis and antitumor activities of novel 2-substituted pyrimidinone-5-carboxylic acid benzylamides

Abstract The title compounds were synthesized via *N*-benzylmalonamic acid methyl ester (**3**). As the key intermediate, **3** was prepared from methyl malonyl chloride and benzylamine. Then, compound **3** was reacted with dimethylformamide dimethyl acetal yielding vinylogue amides **4** and **5**. Isomers **4** and **5** were respectively treated with amidine and guanidine to afford the title compounds 2-substituted pyrimidinone-5-carboxylic acid benzylamides **6** and **7**. All of the new compounds were characterized by ^{1}H-NMR (nuclear magnetic resonance), ^{13}C-NMR, MS and High Resolution Mass Spectrometer (HRMS). The antitumor activities of the compounds were tested in vitro against LoVo cells and Hep3B cells. Both compounds **6** and **7** show activity against these two cell lines.

Keywords pyrimidinone, synthesis, antitumor

1 Introduction

A broad range of biological activities has been reported for compounds containing the pyrimidinone ring system, and many of them have interesting therapeutic properties including anticancer, antiviral, central stimulant and calcium antagonist [1,2]. *N*-Substituted pyrimidine-5-formamide compounds show angiotensin II antagonist activity [3]. There are many methods to synthesize pyrimidinones [4,5], in which the Biginelli reaction is frequently used [6].

After synthesizing the quinazolin-4-ones, which contain a skeleton of pyrimidinone [7], we developed a program to synthesize substituted pyrimidinone compounds. Formamide acetal is often used in the synthesis of pyrimidine rings [8]. In this paper, *N,N*-Dimethylformamide dimethyl acetal (DMF-DMA) was reacted with *N*-benzylmalonamic acid methyl ester. The formed product was then reacted with guanidine or amidine to afford two new pyrimidinone derivatives. Their antitumor activities were tested in vitro against cancer cells.

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2 Experimental

2.1 Instruments and reagents

All reagents and solvents were purchased as reagent grade, and were purified and dried by standard methods where needed. Dichloromethane and ethyl acetate were distilled from CaH_2. All organic solvents were evaporated under reduced pressure with a rotary evaporator. The plates used for thin-layer chromatography (TLC) were E. Merck silica gel $60F_{254}$ (0.1 mm thickness) pre-coated on aluminum plates, and were visualized under ultraviolet (UV) light. Compounds on TLC plates were visualized with a spray of 5% dodecamolybdophosphoric acid in ethanol and with subsequent heating. Flash column chromatography was performed on silica gel (200–300 mesh) from Qingdao Haiyang Chemicals Factory. Dulbecco's modified Eagle's medium (DMEM) is from Gibco Company. Culture medium was prepared freshly before use. Newborn calf serum (FCS) was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., LTD. Trypsin was purchased from Gibco Company; 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) and pyridinium iodide (PI) were purchased from Sigma Chemical Company.

Melting points were measured by using a SGW X-4 microscope melting point analysis instrument and were uncorrected. Nuclear magnetic resonance (NMR), spectra were recorded on a Bruker DPX-300 Spectrometer. Mass spectra (Energy Resolved Mass Spectrum (ERMS) and High Resolution Mass Spectrometer (HRMS)) were obtained with a Thermofinnigan MAT95XL spectrometer or API 2000 LC/MS/MS system. CO_2 incubator: American Forma Scientific Company; NIKON phase-contrast microscope: Japan; Model 680 microplate reader: American Bio-Rad Company.

2.2 Synthesis of substituted pyrimidinones

Two new substituted pyrimidinones **6** and **7** were synthesized as illustrated in Scheme 1.

Synthesis of compounds **6** and **7** were accomplished by two different strategies (Scheme 1). Methyl malonyl chloride (**1**) was treated with benzylamine to give intermediate *N*-benzyl-malonamic acid methyl ester **3**. The latter was then reacted with dimethylformamide dimethyl acetal to yield the vinylogous amide 2-benzylcarbamoyl-3-dimethylamino-acrylic acid methyl ester, which exists in two stereoisomers, i.e., the *Z*- and *E*-isomers **4** and **5**. Compounds **4** and **5** were then reacted with amidine and guanidine, respectively, to afford the targeted pyrimidinones **6** and **7**.

2.2.1 *N-Benzyl malonamic acid methyl ester* (*3*)

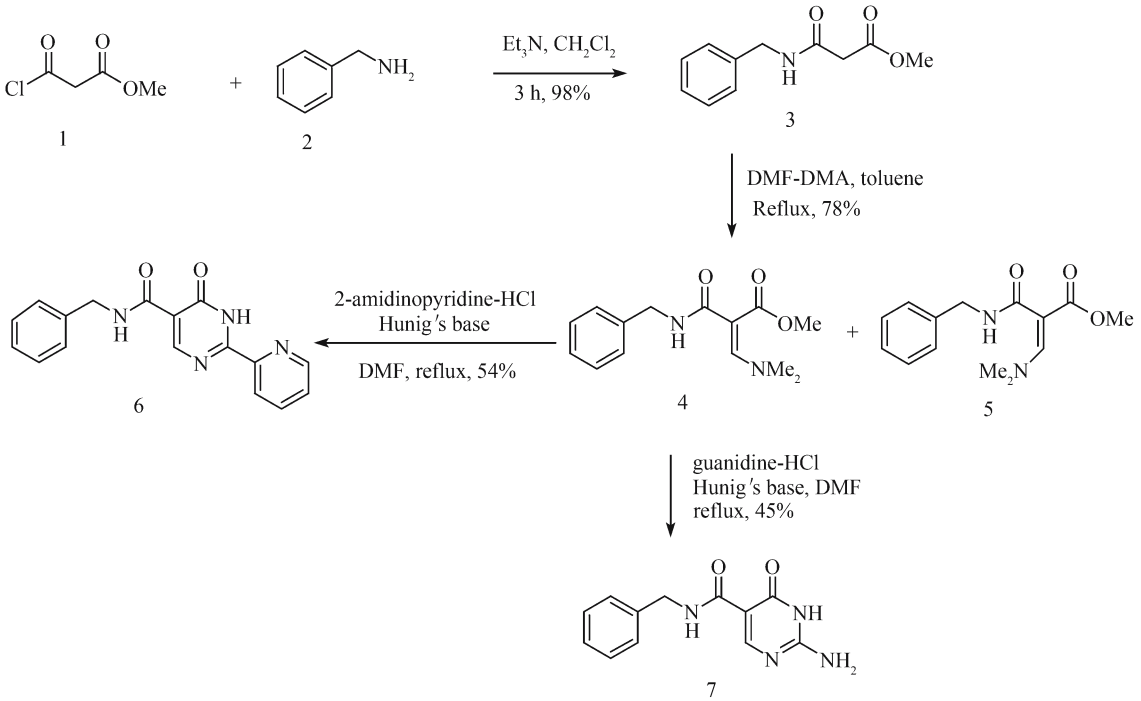
Methyl malonyl chloride (16.6 mL, 150 mmol, 1.0 eq) was added dropwise over 30 min to a 0.30 M benzylamine solution (16.4 mL, 150 mmol, 1.0 eq) and triethylamine (23 mL, 165 mmol, 1.1 eq) in anhydrous CH_2Cl_2 (500 mL). The reaction mixture was stirred for 3 h at ambient temperature, and was then diluted with CH_2Cl_2 (500 mL). The solution was washed sequentially with saturated NH_4Cl and brine (300 mL). The solution was then dried over Na_2SO_4, filtered, and concentrated to give 30.5 g product (98% yield). ^{1}H NMR ($CDCl_3$) δ: 3.23 (s, 2H, $-CH_2-$), 3.62 (s, 3H, O–CH_3), 4.33 (d, J = 5.7, 2H, C_6H_5–CH_2–), 7.16–7.27 (m, 5H, C_6H_5–), 7.67 (s, br, 1H, –NH–); ^{13}C NMR ($CDCl_3$) δ: 41.1, 43.1, 52.0, 127.0, 127.2, 128.2, 137.7, 165.1, 169.0; MS *m/z*: 208 $[M+H]^+$.

2.2.2 *2-Benzylcarbamoyl-3-dimethylamino-acrylic acid methyl ester* (*4*)

Dimethylformamide dimethyl acetal (30 mL, 223.5 mmol, 1.5 eq) was added to a solution of compound **3** (150 mmol, 1.0 eq) in anhydrous toluene (500 mL). The reaction was refluxed for 15 h and was then cooled to ambient temperature. Volatiles were removed under reduced pressure. The material was purified by flash chromatography (EtOAc/MeOH, 97:3) to give brown oil (30 g, 78% yield). ^{1}H-NMR ($CDCl_3$) δ: 3.08 (s, br, 6H, $-N(CH_3)_2$), 3.68 (s, 3H, O–CH_3), 4.51 (d, J = 5.7, 2H, C_6H_5–$\underline{CH_2}$–), 7.27 (m, 5H, C_6H_5–), 7.73 (s, br, 0.58H, =C–H(Z)), 8.20 (s, br, 0.42H, =C–H(E)),7.96, (s, br, 0.66H, –NH–), 8.47 (s, br, 0.34H, –NH–); ^{13}C NMR ($CDCl_3$) δ: 42.4, 50.0, 91.7(89.1), 126.0, 126.6, 127.6, 138.7, 155.4, 157.8, 158.6, 165.3, 168.0; MS *m/z*: 263 $[M+H]^+$.

2.2.3 *6-Oxo-2-pyridin-2-yl-1,6-dihydro-pyrimidine-5-carboxylic acid benzylamide* (*6*)

Diisopropylethylamine (Hünig's base) (7.0 mL, 40 mmol, 2 eq) and 2-amidinopyridinium chloride (6.3 g, 40 mmol, 2 eq) were added to a solution of compound **4** (5.2 g, 20 mmol, 1 eq) in *N,N*-Dimethylformamide (DMF) (75 mL). The reaction was refluxed for 18 h and then cooled to ambient temperature. Volatiles were removed under reduced pressure. Water was added (100 mL) to the residue and then filtered. The solid was washed with H_2O (50 mL) and ethyl acetate (50 mL). The material was purified by flash chromatography (CH_2Cl_2-MeOH 98:2). A tan solid (3.3 g, 54%) was obtained. ^{1}H NMR ($CDCl_3$) δ: 4.67 (d, J = 6, 2H, Ph–CH_2–), 7.26–7.36



Scheme 1 Synthesis of 2-substituted prymidinone-5-carboxylic acid benzylamide

(m, 5H, C_6H_5–), 7.54 (m, 1H), 7.94 (m, 1H), 8.53 (d, $J = 9$, 1H), 8.68 (d, J = 6, 1H), 9.13 (s, 1H), 9.47 (s, br, 1H), 11.38 (s, br, 1H); ^{13}C NMR ($CDCl_3$) δ: 43.4, 118.0, 123.1, 127.4, 127.7, 128.6, 138.3, 146.6, 149.2, 155.8, 159.5, 161.0, 162.6; MS *m/z*: 307 (MH^+); HRMS (FAB) Calcd for $C_{17}H_{15}N_4O_2$ $[M+H]^+$: 307.1190. Found: 307.1198.

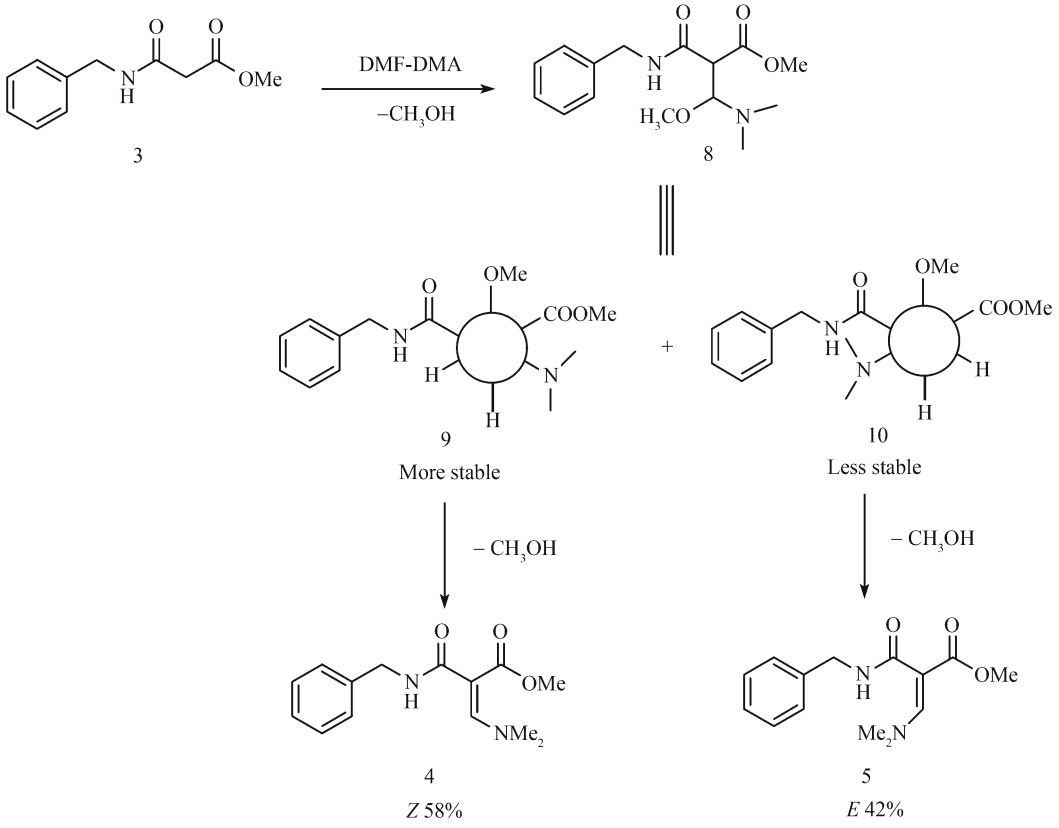
2.2.4 *2-Amino-6-oxo-1,6-dihydro-pyrimidine-5-carboxylic acid benzylamide* **(*7*)**

Diisopropylethylamine (52 mL, 300 mmol, 2.0 eq) and guanidine hydrochloride (29 g, 300 mmol, 2.0 eq) were added to a solution of compound **4** (40 g, 150 mmol, 1.0 eq) in DMF (450 mL). The reaction was refluxed for 15 h and then allowed to cool to ambient temperature. Volatiles were removed under reduced pressure. To the residue was added H_2O (200 mL). The mixture was stirred for 1 h, filtered and washed consecutively with H_2O (100 mL), ethanol (50 mL) and CH_2Cl_2 (50 mL). After drying in vacuo, a grey white solid (16.5 g, 45%) was obtained. ^{1}H NMR ($CDCl_3$) δ: 4.42 (d, $J = 6$, 2H, Ph–CH_2–), 6.15 (s br, 2H, –NH_2), 7.18–7.32 (m, 5H, C_6H_5–), 8.28 (s, 1H), 8.48 (s, 1H), 10.59 (t, $J = 6$, 1H), 11.38 (s, br, 1H); ^{13}C NMR ($CDCl_3$) δ: 41.8, 104.3, 126.8, 127.4, 128.5, 140.6, 159.5, 164.5, 166.8, 172.5; MS *m/z*: 245 (MH^+); HRMS (FAB) Calcd for $C_{12}H_{13}N_4O_4$ $[M+H]^+$: 245.1033. Found: 245.1037.

2.3 Antitumor activity evaluations in vitro

The new compounds **6** and **7** were evaluated against human cancers including the colon LoVo, a human colon carcinoma cell line, and Hep3B cells, a human hepatoma cell line. Drugs were dissolved in diluted hydrochloric acid (0.1 mol/L). Phosphate-buffered saline (PBS) was added to this solution to make a final volume of 50 mL. The solution was sterilized by filtration with a 0.22 μm microporous membrane, and then diluted to the desired concentrations. LoVo and Hep3B cells were cultured in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL of penicillin G and 100 mg/mL of streptomycin. Cells were incubated at 37°C in the 5% CO_2 incubator.

When the cells grew to the exponential growth period, they were digested with 0.25% trypsin, and were then centrifuged for 5 min at 1 000 r/min. The precipitated cells were adjusted to 2×10^4 and 3×10^4 cells/mL with culture medium. Cells were seeded at 160 μL each well in 96-well plates. The cells were incubated for 24 h at 37°C until substratum attachment. Drugs were added to a final volume of 200 μL. There were seven wells for each drug concentration. After 48 h, 20 μL of MTT (5 mg/mL) was added, and the cells were incubated for another 4 h. Medium was removed, and the cells were washed with normal saline twice. The cells were re-suspended in 150 μL of DMSO, and the absorbance



Scheme 2 Mechanism of formation of *Z*- and *E*-isomers

was measured at 630 nm with a microplate reader. Each assay was performed in triplicate. Inhibition of cell proliferation was calculated.

Inhibition rate (%) = $(1 - OD_{sample}/OD_{control}) \times 100\%$

Statistical analysis and IC_{50} values of the drugs were performed with Pharmacologic Calculation System-Version 4.1 software.

3 Results and discussion

3.1 Synthesis of substituted pyrimidinones

Some similar structures of pyrimidinones were found in patents [11,12], but same compounds as **6** and **7** were not found by search using SciFinder Scholar 2006, in which the 2-phenyl-6-oxo-1,6-dihydro-pyrimidine-5-carboxylic acid benzylamide and 2-dimethylamino-6-oxo-1,6-dihydro-pyrimidine-5-carboxylic acid benzylamide were used as the intermediates. However, the ring closure of pyrimidine was not reported in these literatures. The ring closure methods of pyrimidines were reviewed by Abdulla et al [8], in which DMF-DMA condensated with α-position of carbonyl compounds, then the products reacted with guanidine to give the ring closure of pyrimidines. Similar yields were obtained in this paper.

Synthesis of the key intermediate 2-benzylcarbamoyl-3-dimethylamino-acrylic acid methyl ester is the most important step in the synthesis of the pyrimidine derivatives for the product of this reaction is a pair of geometrical isomers namely the *Z*-isomer **4** and E-isomer **5**. Based on ^{1}H NMR analysis of the mixture, it was calculated that the Z-isomer was 58%, and the E-isomer 42%. Because only the Z-isomer **4** can be utilized for further reaction, almost half of the product is wasted. We have not yet succeeded in improving the yield of **4** despite numerous efforts.

Why is the yield of the Z-isomer higher than that of the *E*-isomer? It was hypothesized that the reaction follows the mechanism as shown in Scheme 2 [9].

3.2 Primary evaluation of anticancer activity of new compounds

The antitumor activity of the compounds were tested in vitro against LoVo cells, a human colon carcinoma cell line, and Hep3B cells, a human hepatoma cell line, and the results are shown in Table 1. Both compounds **6** and **7** have modest activity against the two human cancer cell lines. Compound **7** is more potent than **6**, especially in Hep3B cells, a liver cancer which is hard to treat. Compounds **6** and **7** are less potent than nolatrexed (chemical name: 2-amino-6-methyl-5-(pyridin-4-ylsulfanyl) -3*H*-quinazolin-4-one dihydrochloride), a compound containing a similar chemical structure of 2-aminopyrimidin-4-one [10]. We presumed that 2-aminopyrimidine-4-one may be the pharmacophore of this class of antitumor agents.

Table 1 IC_{50} of compound 6 or 7 against LoVo and Hep3B

Compound	IC_{50} / (μmol/L)	
	LoVo	Hep3B
6	16.70 (13.05~20.35)	25.91 (15.55~36.26)
7	11.93 (8.35~15.51)	4.99 (2.83~7.15)

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