Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Medicinal Chemistry 38 (2003) 525–532
EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY
www.elsevier.com/locate/ejmech

Short communication

Synthesis and biological evaluations of pyrazolo[3,4-*d*]pyrimidines as cyclin-dependent kinase 2 inhibitors

Dong Chan Kim [a], Yeo Ran Lee [a], Beom-Seok Yang [a], Kye Jung Shin [a], Dong Jin Kim [a], Bong Young Chung [b,*], Kyung Ho Yoo [a,*]

[a] *Medicinal Chemistry Research Center, Korea Institute of Science and Technology, Seoul 130-650, Republic of Korea*
[b] *Department of Chemistry, Korea University, Seoul 136-701, Republic of Korea*

Received 2 September 2002; received in revised form 31 October 2002; accepted 10 January 2003

Abstract

A series of 1,4,6-trisubstituted pyrazolo[3,4-*d*]pyrimidines **15**–**19**, **30**–**38** capable of selectively inhibiting CDK2 activity were synthesized by derivatization at C-4, C-6 and N-1 with various amines and lower alkyl groups. For above synthetic compounds, biological evaluation was carried out and structure–activity relationship was examined. In our series, 4-anilino compounds exhibited better CDK2 inhibitory activity and antitumor activity compared to 4-benzyl compounds. The compounds **33a**,**b** having a 3-fluoroaniline group at C-4 showed comparable or superior CDK2 inhibitory activity to those of olomoucine and roscovitine as reference compounds. In general, the unsubstituted compounds (**30a**,**b**, **33a**,**b**, **36a**,**b**) at N-1 possessed higher potency than the substituted compounds (**32a**,**b**, **34a**,**b**) for the CDK2 inhibitory activity. As for EGFR inhibitory activity, most compounds didnot have a significant activity. The compounds **32a**,**b** exhibited potent cell growth inhibitory activity against human cancer cell lines, but their CDK2 inhibitory activities were slightly poorer than olomoucine.

Keywords: CDK; Kinase inhibitors; Pyrazolo[3,4-*d*]pyrimidines; CDK2 inhibitory activity; Cell growth inhibitory activity

1. Introduction

It is becoming clear that many types of cancer are the result of abnormal signal-transducing proteins which lead to continual production of the signal for cell division. The mutated genes that encode these defective signaling proteins are oncogenes. They were originally discovered in tumor-causing viruses. A variety of human tumor cells have been shown to produce their own growth factors [1]. The central role of tyrosine phosphorylation in cell proliferative signaling mechanisms provides another target for chemotherapy. It suggests that a specific tyrosine kinase inhibitor would have significant therapeutic potential as an antitumor agent [2–4].

Currently, 2,4,9-trisubstituted purines [5–11] and quinazolines [12–15] exhibit a potent inhibitory effect on the protein kinases. Cyclin-dependent kinases (CDKs) have recently raised considerable interest as the principal regulators of the cell division cycle [16–22]. Olomoucine [23–26] and roscovitine [27,28] (Fig. 1), the representative compounds of purine derivatives, exhibit moderate inhibitory activities ($IC_{50} = 7$ and 0.5 μM) but good selectivity for CDK1/cyclin B1, CDK2/cyclin A, CDK2/cyclin E, CDK5/p25, and CDK7/cyclin H of CDKs over other CDKs or other kinases.

In this work, we carried out the chemical modification by the introduction of pyrazole ring instead of imidazole ring on purine to give a specific binding mode different from the olomoucine analogues [6,29–31]. To this end, we synthesized 1,4,6-trisubstituted pyrazolo[3,4-*d*]pyrimidines **15**–**19**, **30**–**38** (Fig. 1) by introducing a variety of the substituent groups at C-4, C-6 and N-1 of pyrazolopyrimidine ring for a potent and selective inhibitor of the CDK2 by competitive binding at the ATP site, and evaluated for their CDK2, EGFR inhibitory activities and cell growth inhibitory activities.

* Corresponding auther.
E-mail address: khyoo@kist.re.kr (K.H. Yoo).

0223-5234/03/$ - see front matter
doi:10.1016/S0223-5234(03)00065-5

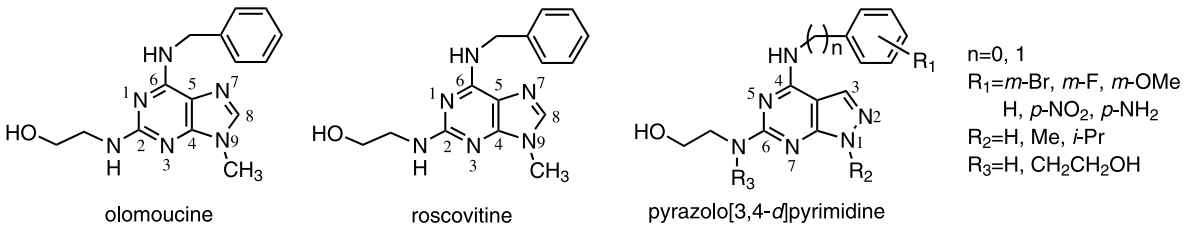

Fig. 1. Structure of olomoucine, roscovitine, and pyrazolo[3,4,*d*]pyrimidines.

2. Chemistry

4-Chloro-6-methylmercaptopyrazolo[3,4-*d*]pyrimidine (**6**) [32], as a key intermediate for the synthesis of title compounds, was synthesized starting from ethoxymethylenemalononitrile (**1**) by the sequence of reactions shown in Fig. 2.

This synthesis was performed in a process that the pyrazole ring was first formed and the pyrimidine ring formation was followed. In pyrazolopyrimidine ring system, the chloro substituent as a leaving group was introduced at C-4 which was the most reactive site to nucleophilic attack. And the methylmercapto group was employed at C-6 as a precursor to introduce later the methylsulfonyl group which is five times more reactive than the chloro substituent [33].

Ethoxymethylenemalononitrile (**1**) was cyclized by nucleophilic substitution of hydrazine to give 3-amino-4-cyanopyrazole (**2**) in 84% yield, which was converted to 3-amino-4-pyrazolecarboxamide hydrosulfate (**3**) by hydrolysis of the nitrile group with 95% sulfuric acid in 88% yield. Subsequent fusion of **3** with thiourea provided 4-hydroxy-6-mercaptopyrazolo[3,4-*d*]pyrimidine (**4**) in moderate yield. **4** was alkylated with iodomethane and sodium hydroxide at 5 °C to afford 4-hydroxy-6-methylmercaptopyrazolo[3,4-*d*]pyrimidine (**5**) in quantitative yield. Chlorination of **5** with phosphorus oxychloride in the presence of *N*,*N*-dimethylaniline yielded 4-chloro-6-methylmercaptopyrazolo[3,4-*d*]pyrimidine (**6**) in 92% yield.

1,4,6-Trisubstituted pyrazolopyrimidines **15**–**19** with a variety of the substituent groups at N-1, C-4, and C-6 positions were prepared as shown in Fig. 3.

Treatment of **6** with benzylamines **7** in the presence of Hunig's base in *n*-butanol resulted in 4-(4′-substituted benzylamino)-6-methylmercaptopyrazolo[3,4-*d*]pyrimidines (**8**) by nucleophilic displacement of the chlorine atom, respectively. N-1 substituted pyrazolo[3,4-*d*]pyrimidines **10**, **11** were prepared by *N*-alkylation of **8** with the appropriate alcohols **9** using Mitsunobu condition [34] in 86–96% yields. Then, in order to introduce the amino group at C-6 position of **8**, **10**, **11**, oxidation of methylmercapto groups using *m*-CPBA and followed by nucleophilic displacement of the resulting activated methylsulfonyl groups with hydroxylamines **14** led to the corresponding 1,4,6-trisubstituted pyrazolo[3,4-*d*]pyrimidines **15**–**18**. Compounds **18** bearing 4-nitrobenzyl moiety at C-4 were carefully reduced with Pd/C in methanol to yield **19** of 4-aminobenzyl moiety.

On the other hand, compounds **30**–**38** were prepared from **6** (Fig. 4), by means of reactions such as amination at C-4 by anilines **20**, alkylation at N-1 using Mitsunobu condition, oxidation of methylmercapto groups with *m*-CPBA, and nucleophilic substitution of activated sulfones with hydroxylamines **29** in a mixture of *n*-BuOH and DMSO.

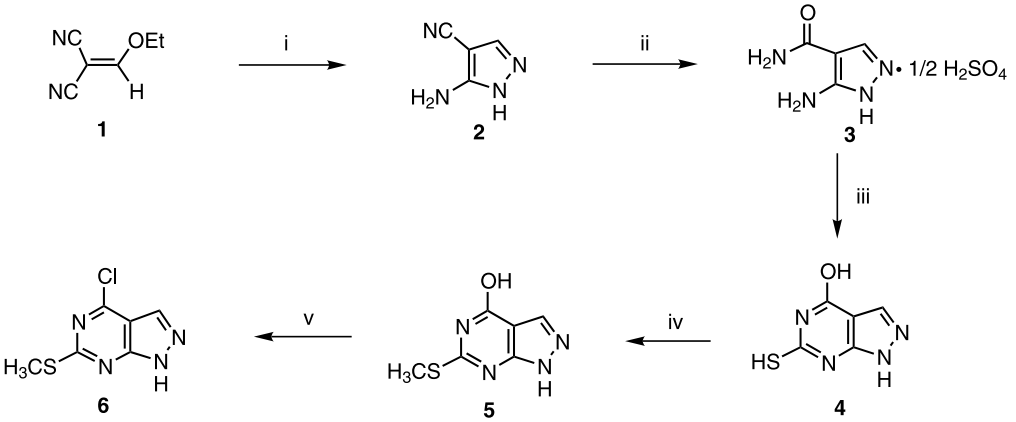

Fig. 2. Reagents and reaction conditions: (i) $H_2NNH_2 \cdot H_2O$ (84%); (ii) 95% H_2SO_4 (88%); (iii) thiourea (70%); (iv) CH_3I, NaOH (99%); (v) $POCl_3$, $PhNMe_2$ (92%).

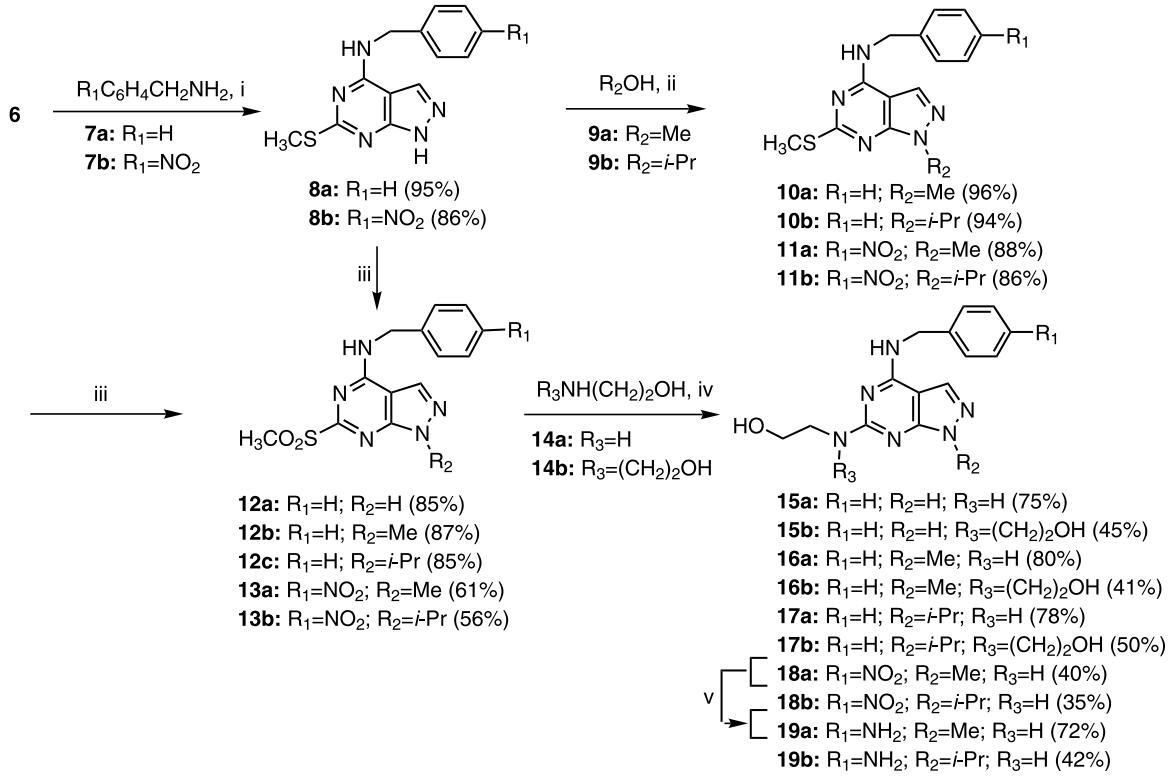

Fig. 3. Reagents and reaction conditions: (i) Hunig's base, *n*-BuOH; (ii) PPh_3, DEAD, THF–CH_2Cl_2 (1:1); (iii) *m*CPBA, CH_2Cl_2, 0 °C; (iv) *n*-BuOH–DMSO (4:1); 90 °C, 4 h; (v) Pd/C, MeOH.

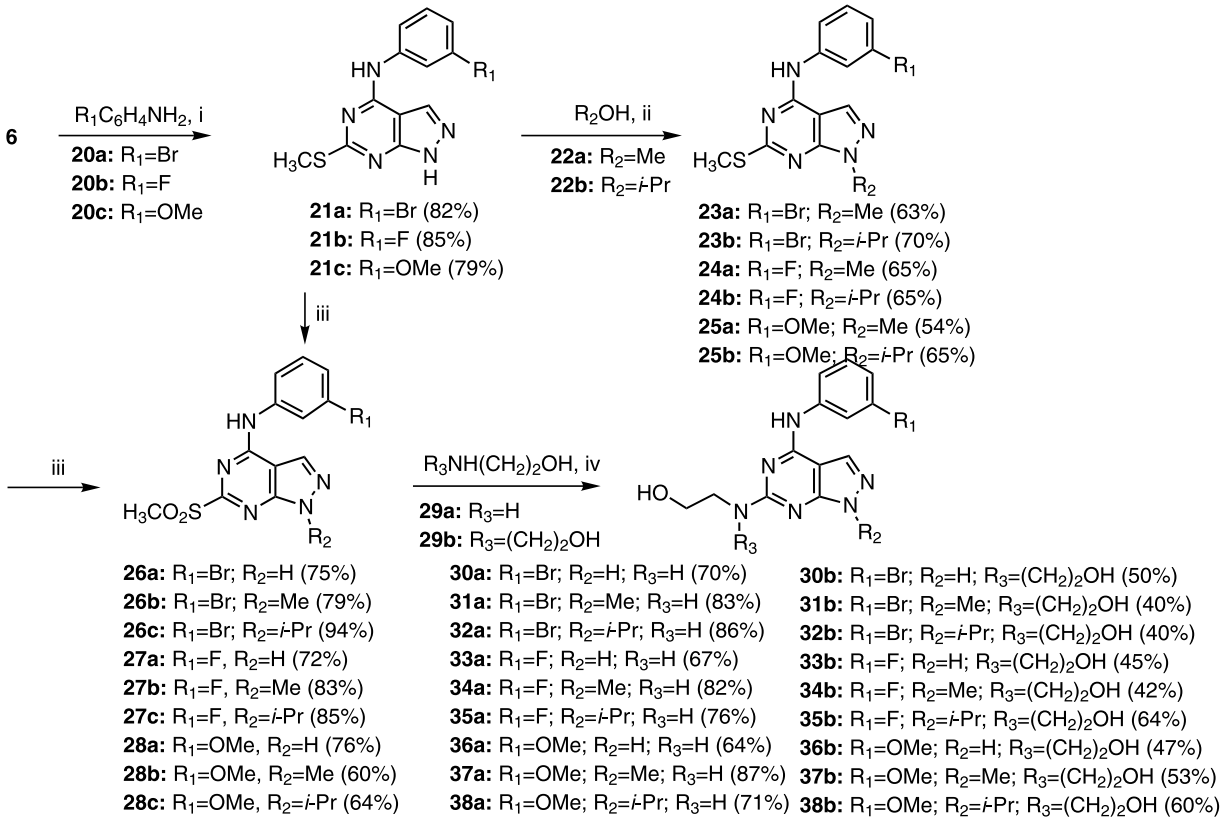

Fig. 4. Reagents and reaction conditions: (i) Hunig's base, *n*-BuOH; (ii) PPh_3, DEAD, THF–CH_2Cl_2 (1:1); (iii) *m*CPBA, CH_2Cl_2, 0 °C; (iv) *n*-BuOH–DMSO (4:1), 90 °C, 4 h.

Table 1
Physical properties and spectral data of the selected intermediates

Compound	M.p. (°C)	^{1}H-NMR (solvent: δ, ppm)	^{13}C-NMR (δ, ppm)
8a	212–214	DMSO-d_6: 8.77 (t, 1H, $J = 5.7$ Hz), 8.09 (s, 1H), 7.37–7.23 (m, 5H), 4.71 (d, 2H, $J = 5.7$ Hz), 2.44 (s, 3H)	168.9, 156.0, 153.9, 139.9, 133.3, 129.1, 128.2, 127.7, 98.5, 43.9, 14.3
8b	208–210	DMSO-d_6: 13.32 (s, 1H), 8.92 (t, 1H), 8.21 (d, 2H, $J = 8.6$ Hz), 8.06 (s, 1H), 7.60 (d, 2H, $J = 8.6$ Hz), 4.82 (d, 2H, $J = 5.7$ Hz), 2.39 (s, 3H)	169.0, 156.1, 148.4, 147.3, 133.4, 129.1, 124.1, 98.6, 43.6, 14.4
10a	94–95	DMSO-d_6: 7.71 (s, 1H), 7.36–7.31 (m, 5H), 4.81 (d, 2H), 3.94 (s, 3H), 2.58 (s, 3H)	167.8, 154.8, 152.9, 138.7, 131.2, 127.9, 127.1, 126.5, 97.6, 42.8, 32.8, 13.1
10b	166–167	$CDCl_3$: 7.75 (s, 1H), 7.38–7.28 (m, 5H), 5.09 (m, 1H, $J = 6.6$ Hz), 4.83 (d, 2H, $J = 5.5$ Hz), 2.60 (s, 3H), 1.54 (d, 6H, $J = 6.6$ Hz)	169.0, 156.1, 153.5, 138.1, 133.5, 129.3, 128.2, 127.7, 98.7, 49.1, 45.5, 22.3, 14.6
12a	205–208	DMSO-d_6: 9.43 (s, 1H), 8.30 (s, 1H), 7.40–7.27 (m, 5H), 4.78 (s, 2H), 3.29 (s, 3H)	163.6, 157.5, 154.5, 139.3, 133.6, 129.3, 128.7, 128.1, 101.3, 46.7, 39.8
12b	110–115	DMSO-d_6: 9.46 (s, 1H), 8.25 (s, 1H), 7.41–7.27 (m, 5H), 4.78 (d, 2H, $J = 5.1$ Hz), 3.95 (s, 3H), 3.30 (s, 3H)	163.7, 157.5, 152.8, 139.4, 133.2, 129.5, 128.8, 128.4, 101.7, 44.9, 40.1, 35.0
12c	122–124	$CDCl_3$: 7.98 (s, 1H), 7.37–7.27 (m, 5H), 5.19 (m, 1H, $J = 6.6$ Hz), 4.87 (d, 2H, $J = 5.6$ Hz), 3.31 (s, 3H), 1.54 (d, 6H, $J = 6.6$ Hz)	162.7, 157.3, 151.7, 137.9, 131.5, 129.1, 128.4, 127.3, 101.9, 49.8, 45.6, 39.5, 22.4
21a	> 250	DMSO-d_6: 10.33 (s, 1H), 8.29 (s, 1H), 8.31–7.29 (m, 4H), 2.54 (s, 3H)	168.0, 155.4, 153.0, 140.7, 132.3, 130.5, 125.7, 123.1, 121.3, 119.3, 98.4, 13.6
21b	242–247	DMSO-d_6: 10.19 (s, 1H), 8.22 (s, 1H), 7.93–6.87 (m, 4H), 2.51 (s, 3H)	168.9, 164.4, 161.2, 156.2, 153.7, 141.7, 141.5, 133.2, 131.0, 130.8, 117.0, 110.4, 110.1, 108.4, 108.0, 99.1, 14.4
21c	218–222	DMSO-d_6: 10.04 (s, 1H), 8.21 (s, 1H), 7.60–6.70 (m, 4H), 3.80 (s, 3H), 2.54 (s, 3H)	169.2, 160.5, 156.4, 154.2, 141.3, 133.7, 130.5, 114.1, 109.9, 107.7, 99.3, 56.0. 14.7
23a	141–144	DMSO-d_6: 10.22 (s, 1H), 8.19 (s, 1H), 8.30–7.27 (m, 4H), 3.89 (s, 3H), 2.57 (s, 3H)	168.3. 153.6, 152.9, 140.6, 131.6, 130.6, 125.7, 123.2, 121.4, 119.3, 98.8, 33.4, 13.8
23b	110–115	$CDCl_3$: 7.89 (s, 1H), 7.49–7.26 (m, 4H), 5.08 (m, 1H, $J = 6.7$ Hz), 2.64 (s, 3H), 1.52 (d, 6H, $J = 6.7$ Hz)	167.7, 154.0, 153.4, 139.1, 132.0, 131.0, 129.4, 127.1, 123.2, 122.5, 98.6, 49.4, 22.3, 14.7
26a	208–210	DMSO-d_6: 7.90 (s, 1H), 8.12–7.15 (m, 4H), 3.30 (s, 3H)	163.0, 155.0, 153.0, 141.0, 139.5, 134.0, 127.6, 124.6, 122.3, 120.7, 102.4, 40.2
26b	> 260	DMSO-d_6: 10.73 (s, 1H), 8.34–7.33 (m, 5H), 3.99 (s, 3H), 3.41 (s, 3H)	163.0, 155.0, 153.0, 140.8, 133.2, 131.8, 127.7, 124.6, 122.5, 120.8, 102.6, 40.2, 35.1
26c	200–204	$CDCl_3$: 8.39 (s, 1H), 7.36–6.80 (m, 4H), 5.06 (m, 1H, $J = 6.7$ Hz), 3.33 (s, 3H), 1.42 (d, 6H, $J = 6.7$ Hz)	164.9, 162.2, 161.6, 152.2, 139.2, 132.4, 130.8, 119.3, 113.1, 110.7, 101.9, 49.9, 39.7, 22.3

Physical properties and spectral data of the selected compounds are given Tables 1 and 2.

3. Biological results and discussion

CDK2 and EGFR inhibitory activities of pyrazolo[3,4-*d*]pyrimidines **15**–**19**, **30**–**38** prepared above were shown in Table 3, together with those of olomoucine, roscovitine and PYK2104 as reference compounds. CDK2 is one of CDK family protein which has a proline directed serine/threonine kinase activity, phosphorylating serine or threonine residue ahead of proline, whereas EGFR belongs to receptor tyrosine kinase family. Compounds were further evaluated for their cell division inhibitory activities against three human tumor cell lines. These tumor cells divide at least twice for 2-day period of the cell division inhibition test in the absence of inhibitory compounds and the kinase activity of CDK2/cyclin A is indispensable for the division of these cells.

In general, 4-anilino compounds **30**–**38** showed better CDK2 and cell division inhibitory activities than the corresponding 4-benzyl analogues **15**–**19**. The compounds **33a**,**b** having a 3-fluoroaniline group at C-4 were as active as roscovitine and much more active than olomoucine as reference compounds, whereas they possessed poor cell division inhibitory activity. The unsubstituted compounds (**30**, **33**, **36**) at N-1 exhibited higher potency compared to the substituted compounds (**31**, **32**, **34**, **35**, **37**, **38**) for the CDK2 inhibitory activity. As a nitrogen atom as a hydrogen bond acceptor in pyrazolopyrimidine scaffold is missing at C-3 position equivalent to N-7 position of olomoucine, pyrazolopyrimidine ring system has to flip for hydrogen bonding with the protein hinge region. To this reason, the unsubstituted compounds at N-1 are more active owing to the possibility of an additional hydrogen bonding by ring flip (Fig. 5).

In the correlation of the CDK2 inhibitory activity with the substituent size at C-6 of pyrazolopyrimidine skeleton, there was no significant difference between mono-ethanolamine and branched di-ethanolamine compounds. Most compounds did not show any significant EGFR inhibitory activity, indicating good selectivity in the situation of protein kinase inhibitors.

Table 2
Physical properties and spectral data of the selected pyrazolopyrimidines

Compound	M.p. (°C)	^{1}H-NMR (solvent: δ, ppm)	^{13}C-NMR (δ, ppm)/FABHRMS *m/z*
15a	230–234	DMSO-d_6: 12.53 (s, 1H), 8.21 (s, 1H), 7.85 (s, 1H), 7.34–7.23 (m, 5H), 6.39 (s, 1H), 4.67 (d, 2H), 3.51–3.31 (m, 4H)	162.4, 158.2, 157.3, 140.8, 133.1, 129.1, 128.3, 127.6, 96.0, 61.2, 44.5, 43.7
15b	242–245	DMSO-d_6: 8.32 (s, 1H), 7.83 (s, 1H), 7.37–7.23 (m, 5H), 4.62 (m, 3H), 3.60–3.46 (m, 8H)	161.3, 158.1, 156.9, 141.0, 133.1, 129.1, 128.3, 127.6, 95.1, 60.4, 52.5, 44.6
16a	94–95	CD_3OD: 7.80 (s, 1H), 7.37–7.20 (m, 5H), 4.72 (d, 2H), 3.77 (s, 3H), 3.70 (t, 2H, $J = 5.4$ Hz), 3.54 (t, 2H, $J = 5.5$ Hz) 3.57 (t, 2H)	162.5, 157.4, 155.5, 142.1, 139.6, 131.5, 128.3, 127.4, 126.9, 61.6, 49.2, 46.2, 32.2
16b	130–135	CD_3OD: 7.79 (s, 1H), 7.36–7.23(m, 5H), 4.70 (d, 2H), 3.77 (s, 11H)	161.9, 157.1, 155.9, 140.0, 131.5, 128.4, 127.4, 127.0, 95.6, 61.8, 52.4, 44.1, 32.1
18a	127–130	$CDCl_3$: 8.18 (d, 2H), 7.64 (s, 1H), 7.49 (d, 2H), 5.78 (bs, 1H), 5.41 (t, 1H), 4.85 (d, 2H), 3.84 (s, 3H), 3.82 (t, 2H), 3.59 (t, 2H)	162.5, 157.4, 155.8, 147.8, 146.7, 130.7, 128.4, 124.3, 96.4, 64.9, 45.4, 44.6, 33.9
18b	155–157	$CDCl_3$: 8.19 (d, 2H), 7.68 (s, 1H), 7.50 (d, 2H), 5.87 (bs, 1H), 5.40 (t, 1H), 4.84 (m, 2H), 4.84 (m, 1H), 3.82 (t, 2H), 3.59 (t, 2H), 1.49 (d, 6H)	161.9, 157.0, 154.2, 147.2, 146.3, 130.3, 128.0, 123.8, 96.3, 64.5, 48.4, 44.9, 44.1, 21.8
30a	>260	DMSO-d_6: 7.85 (s, 1H), 8.22–7.09 (m, 4H), 3.66 (t, 2H), 3.47 (t, 2H)	162.0, 158.5, 154.9, 142.8, 141.3, 133.3, 131.2, 125.7, 121.4, 119.7, 96.6, 61.0, 44.7/Calc. for $C_{13}H_{13}BrN_6O$ $(M+H)^+$ 349.0334, Found 349.0412
30b	254–257	DMSO-d_6: 7.82 (s, 1H), 8.21–7.10 (m, 4H), 3.77 (m, 4H), 3.49 (m, 4H)	160.8, 158.3, 154.2, 142.2, 133.0, 131.2, 125.4, 122.9, 122.1, 119.2, 95.7, 60.3, 52.4/Calc. for $C_{15}H_{17}BrN_6O_2$ $(M+H)^+$ 393.0596, Found 393.0667
31a	173–174	DMSO-d_6: 9.67 (s, 1H), 8.00–6.94 (m, 5H), 4.70 (s, 1H), 3.74 (s, 3H), 3.59 (q, 2H), 3.43 (q, 2H)	161.9, 156.5, 154.8, 142.5, 132.2, 131.4, 125.7, 123.1, 122.3, 119.6, 96.8, 60.9, 44.8, 33.8
31b	218–220	DMSO-d_6: 9.78 (s, 1H), 8.32–7.19 (m, 5H), 4.82 (s, 2H), 3.75–3.72 (m, 11H)	160.9 156.5, 154.4, 142.3, 132.2, 131.4, 125.6, 123.1, 122.3, 119.3, 96.2, 60.8, 52.7, 33.6
33a	>260	DMSO-d_6: 12.79 (s, 1H), 9.69 (s, 1H), 8.14–6.79 (m, 5H), 4.73 (t, 2H), 3.60–3.37 (m, 4H)	164.7, 161.9, 161.5, 158.3, 154.8, 142.8, 142.7, 133.2, 130.9, 130.7, 116.3, 109.4, 109.2, 107.9, 107.5, 96.5, 60.8, 44.6/Calc. for $C_{13}H_{13}FrN_6O$ $(M+H)^+$ 289.1135, Found 289.1201
33b	>260	DMSO-d_6: 12.82 (s, 1H), 9.80 (s, 1H), 8.05 (s, 1H), 7.95–6.81 (m, 4H), 4.01 (s, 1H), 3.71–3.64 (m, 8H)	164.5, 161.3, 161.0, 158.4, 154.4, 142.6, 142.4, 133.2, 131.0, 130.9, 116.4, 109.6, 109.3, 107.7, 107.4, 95.9, 60.5, 52.7/Calc. for $C_{15}H_{17}FN_6O_2$ $(M+H)^+$ 333.1397, Found 333.1475
34a	176–179	CD_3OD: 7.96–6.79 (m, 5H), 3.81 (s, 3H), 3.79–3.60 (m, 4H)	164.4, 161.9, 161.5, 156.5, 154.9, 142.7, 142.6, 132.2, 130.9, 130.8, 116.5, 109.6, 109.3, 107.9, 107.6, 96.8, 60.9, 44.7, 33.7
34b	213–215	DMSO-d_6: 9.81 (s, 1H), 8.03 (s, 1H), 7.90–6.81 (m, 4H), 4.80 (s, 2H), 3.84 (m, 3H), 3.80–3.57 (m, 8H)	164.6, 161.4, 160.9, 156.5, 154.5, 142.5, 142.3, 132.2, 131.0, 130.9, 116.4, 109.7, 109.4, 107.8, 107.4, 96.2, 60.0, 52.5, 33.6
36a	254–256	DMSO-d_6: 9.48 (s, 1H), 8.02 (s, 1H), 7.60-6.59 (m, 5H), 4.72 (s, 1H), 3.77 (s, 3H), 3.55 (t, 2H), 3.36 (s, 2H)	161.1, 159.4, 157.34, 154.2, 141.2, 132.4, 129.2, 112.5, 108.0, 105.9, 95.6, 60.0, 55.0, 43.7/Calc. for $C_{14}H_{16}N_6O_2$ $(M+H)^+$ 301.1335, Found 301.1413
36b	>260	DMSO-d_6: 9.51 (s, 1H), 8.01 (s, 1H), 7.48–6.58 (m, 4H), 4.73 (s, 2H), 3.75 (s, 3H), 3.74–3.62 (m, 8H)	161.2, 160.4, 158.3, 154.6, 141.9, 133.2, 130.2, 113.3, 109.1, 106.5, 95.9, 60.2, 56.0, 52.3/Calc. for $C_{16}H_{20}N_6O_3$ $(M+H)^+$ 345.1597, Found 345.1675
37a	184–188	CD_3OD: 7.89 (s, 1H), 7.53–6.67 (m, 4H), 3.83 (s, 3H), 3.81 (s, 3H), 3.77 (t, 2H), 3.61 (t, 2H)	162.0, 160.3, 156.5, 155.1, 142.0, 132.3, 130.1, 113.5, 109.1, 106.8, 96.8, 60.9, 55.9, 44.7, 33.7
37b	171–175	$CDCl_3$: 9.59 (s, 1H), 8.04 (1H, s), 7.51–6.60 (m, 4H), 4.80 (brs, 2H), 3.78–3.67 (m, 14H)	161.1, 160.4, 156.4, 154.7, 141.8, 132.3, 130.2, 113.3, 109.2, 106.4, 96.2, 60.2, 55.9, 52.3 33.6

The compounds **32a**,**b** exhibited potent cell division inhibitory activity against the target organisms but poor CDK2 inhibitory activity.

It seems that there is not a good correlationship between in vitro CDK2 inhibitory activity and in vivo cell growth inhibition activity among some tested compounds. For example, comparing compound **30b** with **32a** and **32b**, **30b** is more potent than **32a** and **32b** for CDK2 inhibition. However, the degree of in vivo cell growth inhibition is reversed between them. Currently it is not clear for the reason. It may be due to different abilities to internalize inside cells, or it may be resulted from different inhibitory activities against some other cellular targets within cells besides CDK2 that might be critical for cell growth.

The results demonstrate that structural variations at C-4, C-6, and N-1 of pyrazolo[3,4-*d*]pyrimidines might be led to potent inhibitors of the CDKs, and these informations will be helpful for designing new inhibitors.

Table 3
CDK2, EGFR, and cell division inhibitory activities of pyrazolo[3,4-*d*]pyrimidines **15**–**19**, **30**–**38**

Compound	IC_{50} (μM)		GI_{50} (μM)		
	CDK2	EGFR	A431 [a]	SNU638 [b]	HCT116 [c]
15a	29.5	93.3	26.56	20.77	4.50
15b	37.2	55.0	> 100	28.98	93.63
16a	> 100	> 100	42.67	37.08	> 100
16b	> 100	77.6	24.28	25.28	53.44
17a	> 100	> 100	35.60	25.17	57.60
17b	> 100	> 100	21.88	26.11	47.68
18a	17.8	> 100	23.36	24.92	48.52
18b	46.8	> 100	32.02	23.45	77.71
19a	> 100	> 100	58.88	96.75	> 100
19b	> 100	> 100	55.27	38.20	> 100
30a	8.1	> 100	> 100	69.22	> 100
30b	3.8	36.3	> 100	38.88	> 100
31a	> 100	47.9	31.86	18.16	> 100
31b	> 100	> 100	2.94	6.20	16.61
32a	28.2	> 100	2.19	4.28	6.46
32b	29.5	> 100	2.58	4.10	5.06
33a	0.5	66.1	28.85	> 100	> 100
33b	0.9	> 100	54.33	> 100	> 100
34a	21.4	> 100	16.29	15.84	28.24
34b	36.3	29.5	3.94	2.56	8.89
35a	49.0	> 100	7.80	7.57	19.28
35b	47.9	> 100	6.25	7.95	10.88
36a	3.1	> 100	> 100	17.07	38.19
36b	10.2	> 100	> 100	> 100	> 100
37a	> 100	> 100	19.14	21.73	90.44
37b	57.5	> 100	12.94	10.49	17.19
38a	> 100	> 100	13.08	7.55	21.44
38b	66.0	> 100	4.43	7.92	12.56
Olomoucine	7.0	–	16.60	16.62	12.05
Roscovitine	0.5	–	–	–	–
PYK2104	–	0.0008	–	–	–
Doxorubicine	–	–	0.18	–	0.13

[a] A431, human vulvar epidermoid carcinoma cell line.
[b] SNU638, human stomach epitherial carcinoma cell line.
[c] HCT116, human colon epitherial carcinoma cell line.

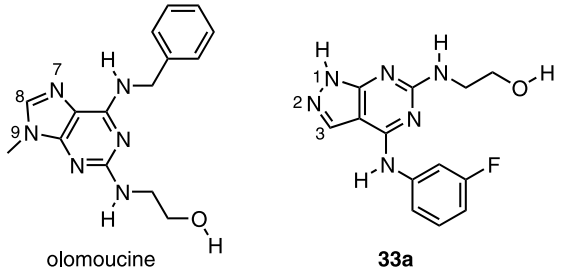

Fig. 5. Comparison of the binding modes of olomoucine with compound **33a**.

4. Experimental

4.1. Chemistry

Melting points were determined on a Thomas–Hoover capillary melting point apparatus and are uncorrected. IR spectra were measured on a Perkin–Elmer 16F PC FT-IR spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a 300 MHz Bruker NMR spectrometer (300 MHz for ^{1}H and 75.5 MHz for ^{13}C) using tetramethylsilane as an internal standard. Mass spectra were obtained on a JEOL SX-102. Microanalytical data were obtained by using a EA 1108 Fisons Instruments. Column chromatography was carried out using silica gel (230–400 mesh). Solvents and liquid reagents were transferred using hypodermic syringes. All other reagents and solvents used were reagent grade.

4.1.1. General procedure for the synthesis of 4-substituted amino-6-methylmercaptopyrazolo[3,4-d]pyrimidines **8** and **21**

To a solution of 4-chloro-6-methylmercaptopyrazolo[3,4-*d*]pyrimidine (**6**, 1.00 g, 5.1 mmol) in *n*-BuOH (80 mL) were added the appropiate amine (**7**, **20**, 5.1 mmol) and diisopropylethylamine (1.60 mL, 9.2 mmol), and the mixture was stirred at 50 °C for 24 h. The mixture was concentrated in vacuo to give the residue, which was diluted with EtOAc and H_2O. The organic layer was dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane–EtOAc = 1.5:1) to give the title compound.

4.1.2. General procedure for the synthesis of 1-alkyl-4-substituted amino-6-methylmercaptopyrazolo[3,4-d]pyrimidines **10**, **11** and **23**–**25**

To a solution of triphenylphosphine (1.30 g, 5.0 mmol) in a mixture of THF (2.50 mL) and CH_2Cl_2 (2.50 mL) was added diethyl azodicarboxylate (0.39 mL, 2.5 mmol) in portions, and the mixture was stirred at 0 °C for 1 h. To a solution of compound **8**, **21** (2.0 mmol) in a mixture of THF (2.5 mL) and CH_2Cl_2 (2.5 mL) was added the appropriate alcohol **9**, **22** (2.0 mmol), followed by the above-prepared solution using cannula at 0 °C, and the mixture was stirred at room temperature (r.t.) for 3 h. When the reaction was completed, the mixture was concentrated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane–EtOAc = 2:1) to afford the title compound.

4.1.3. General procedure for the synthesis of 1-alkyl-4-substituted amino-6-methylsulfonylpyrazolo[3,4-d]pyrimidines **12**,**13**, and **26**–**28**

To a stirred solution of the appropriate compound **10**, **11**, **23**–**25** (1.4 mmol) in CH_2Cl_2 (100 mL) was added 70% solution of 3-chloroperbenzoic acid (0.74 g, 3.0 mmol) at 0 °C for 3 h. After being stirred at r.t. for 3 h, the mixture was concentrated in vacuo to give the residue, which was diluted with EtOAc and saturated aqueous $NaHCO_3$ solution. The organic layer was washed with saturated aqueous $NaHCO_3$ solution,

H_2O and brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (*n*-hexane–EtOAc = 2:1) to give the title compound.

4.1.4. General procedure for the synthesis of 1-alkyl-4-substituted amino-6-substituted hydroxylaminopyrazolo[3,4-d]pyrimidines **15**–**19** and **30**–**38**

To a stirred solution of the appropriate compound **12**, **13**, **26**–**28** (1.0 mmol) in *n*-BuOH (16 mL) and DMSO (4 mL) was added the corresponding ethanolamine **14**, **29** (0.18 mL). After being stirred at 90 °C for 12 h, the mixture was concentrated in vacuo to give the residue, which was diluted with EtOAc. The organic layer was washed with H_2O, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residue was purified by silica gel column chromatography (CH_2Cl_2–MeOH = 20:1) to give the title compound.

4.2. Microbiology methods

4.2.1. Enzymatic activity inhibition assay

The inhibition studies of cell cycle dependent kinase 2 were performed for the synthesized compounds along with olomoucine and roscovitine as reference compounds. Olomoucine was purchased from Sigma (USA) and synthesized roscovitine was kindly provided by Oh et al. [7]. CDK2/cyclin A enzyme was purified from infected sf21 insect cells. For baculoviral over-expressions of proteins, we subcloned human CDK2 c-DNA tagged by hexa-histidine on its N-terminal and human cyclin A c-DNA into pBacPak 8 expression vector (Clon Tech, USA), respectively, and baculovirus which carries each gene was generated using baculovirus generating kit from Clon Tech. CDK2/cyclin A enzyme was purified using Ni^{2+}-affinity resin (Novagen, USA) from sf21 insect cell culture into which CDK2 and cyclin A carrying baculoviruses were cotransfected. Enzyme assays were done in 20 μL of 50 mM Tris–HCl containing 10 μM ATP, 0.2 μCi of gamma-P^{32} ATP, 10 mM $MgCl_2$, 5 mM DTT and 4 μg of histone H1 (Calbiochem, USA) was used as a substrate. The reaction was continued for 10 min in the presence of inhibitors and stopped by adding 10 μL of 30% phosphoric acid. The stopped mixtures were spotted onto P81 paper (Whatman, USA) and were washed with 10 mM Tris–HCl (pH 8.0) containing 0.1 M NaCl five times. The radioactivity of each spot was quantified with BAS imager (Kodak, USA).

The inhibition studies of human EGFR tyrosine kinase activities were done using C-terminal human EGFR tyrosine kinase domain as described previously [35]. PYK2104 provided from Lee was used as a reference compound [35]. The concentration of inhibitor that gives 50% inhibition was designated as IC_{50} value.

4.2.2. Cell growth inhibition assay

Human cancer cell lines, A431 (cervical cancer cell line), SNU638 (stomach cancer cell line), and HCT116 (colon cancer cell line) were obtained from Cell Bank in Medical College of Seoul National University, Seoul, Korea. Cells were grown in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C and 5% CO_2. For cell division inhibition assay, 1000 cells were plated on 96 well plate and next day, test compounds as well as olomucine and doxorubicin (Sigma, USA) as reference compounds were treated to the cells at various concentrations. Cells were allowed to grow further for two days in the presence of compounds, then fixed by adding equal volume of 4% formalin (Sigma) for 30 min. Fixed cells were washed with tap water five times and stained in 0.1% sulforhodamine B (Sigma) for 30 min. Subsequently cells were washed with 1% acetic acid for four times and the dyes attached to cells were eluted by adding 100 μL of 0.1 M Tris–HCl (pH 8.0) and shaking for 10 min. The absorbance was measured at 520 nm wavelength using microplate reader (Molecular Dynamics). The absorbance is proportional to cell number in each well. Measurements were done triplicate and averaged. The absorbance from cells at the time of compound treatment was designated as 0% and the absorbance from cells after two days growth with no compound treatment was assigned as 100%. The GI_{50} value was defined as the inhibitor concentration which gives 50% cell growth inhibition during 2 days period of compound treatment.

Acknowledgements

We are grateful to the Korea Institute of Science and Technology for financial support.

References

[1] S.A. Aaronson, Science 254 (1991) 1146.
[2] L. Meijer, Trends Cell Biol. 6 (1996) 393–397.
[3] L. Meijer, S.-H. Kim, Methods Enzymol. 283 (1997) 113–128.
[4] K.G. Coleman, J.P. Lyssikatos, B.V. Ynag, Annu. Rep. Med. Chem. 32 (1997) 171–179.
[5] N.S. Gray, L. Wodicka, A.-M.W.H. Thunnissen, T.C. Norman, S. Kwon, F.H. Espinoza, D.O. Morgan, G. Barnes, S. Leclerc, L. Meijer, S.-H. Kim, D.J. Lockhart, P.G. Schultz, Science 281 (1998) 533–538.
[6] M. Legraverend, P. Tunnah, M. Noble, P. Ducrot, O. Ludwig, D.S. Grierson, M. Leost, L. Meijer, J. Endicott, J. Med. Chem. 43 (2000) 1282–1292.
[7] C.H. Oh, H.K. Kim, S.C. Lee, C. Oh, B.S. Yang, H.J. Rhee, J.H. Cho, Arch. Pharm. Pharm. Med. Chem. 334 (2001) 345–350.
[8] M. Legraverend, O. Ludwig, S. Leclerc, L. Meijer, J. Heterocycl. Chem. 38 (2001) 299–303.
[9] Y.T. Chang, N.S. Gray, G.R. Rosania, D.P. Sutherlin, S. Kwon, T.C. Norman, R. Sarohia, M. Leost, L. Meijer, P.G. Schultz, Chem. Biol. 6 (1999) 361–375.

[10] S.R. Schow, R.L. Mackman, C.L. Blum, E. Brooks, A.G. Horsma, A. Joly, S.S. Kerwar, G. Lee, D. Shiffman, M.G. Nelson, X. Wang, M.M. Wick, X. Zhang, R.T. Lum, Bioorg. Med. Chem. Lett. 7 (1997) 2697–2702.
[11] P. Imbach, H.G. Capraro, P. Furet, H. Mett, T. Meyer, J. Zimmermann, Bioorg. Med. Chem. Lett. 9 (1999) 91–96.
[12] G.W. Rewcastle, W.A. Denny, A.J. Bridges, H. Zhou, D.R. Cody, A. McMichael, D.W. Fry, J. Med. Chem. 38 (1995) 3482–3487.
[13] A.J. Barker, Eur. Pat. Appl. 566 226 A1, 1993.
[14] J.B. Smail, B.D. Palmer, G.W. Rewcastle, W.A. Denny, D.J. McNamara, E.M. Dobrusin, A.J. Bridges, H. Zhou, H.D.H. Showalter, R.T. Winters, W.R. Leopold, D.W. Fry, J.M. Nelson, V. Slintak, W.L. Elliot, B.J. Roberts, P.W. Vincent, S.J. Patmore, J. Med. Chem. 42 (1999) 1803–1815.
[15] A.J. Bridges, H. Zhou, D.R. Cody, G.W. Rewcastle, A. McMichael, H.D.H. Showalter, D.W. Fry, A.J. Kraker, W.A. Denny, J. Med. Chem. 39 (1996) 267–276.
[16] E.A. Nigg, Trends Cell Biol. 3 (1993) 296–301.
[17] C.S. Sherr, Cell 73 (1993) 1059–1065.
[18] L.H. Hartwell, M.B. Kastan, Science 266 (1994) 1821–1828.
[19] D.O. Morgan, Annu. Rev. Cell Dev. Biol. 13 (1997) 261–291.
[20] X. Grana, E.P. Reddy, Oncogene 11 (1995) 211–219.
[21] D.O. Morgan, Nature 374 (1995) 131–134.
[22] J. Pines, Biochem. J. 308 (1995) 697–711.
[23] J. Vesely, L. Havlicek, M. Strnad, J.J. Blow, A. Donella-Deana, L. Pinna, D.S. Letham, J. Kato, L. Detivaud, S. Leclerc, L. Meijer, Eur. J. Biochem. 224 (1994) 771–786.
[24] L. Meijer, A. Borgne, O. Mulner, J.P.J. Chong, J.J. Blow, N. Inagaki, M. Inagaki, J.-G. Delcros, J.-P. Moulinoux, Eur. J. Biochem. 224 (1994) 771–786.
[25] C.W. Parker, B. Entsch, D.S. Letham, Phytochemistry 25 (1986) 303–310.
[26] R.T. Abraham, M. Axquarone, A. Andersen, A. Asensi, R. Bellé, F. Berger, C. Bergounioux, G. Brunn, C. Buquet-Fargot, D. Fagot, N. Glab, H. Goudeau, M. Goudeau, P. Guettier, P.J. Houghton, H. Hendriks, B. Kloareg, M. Lippai, D. Marie, B. Maro, L. Meijer, J. Mester, O. Mulner-Lorillon, S.A. Poulet, E. Schierenberg, B. Schutte, D. Vaulot, M.H. Verlhac, Biol. Cell 83 (1995) 105–120.
[27] L. Meijer, A. Borgne, O. Mulner, J.P.J. Chong, J.J. Blow, N. Inagaki, M. Inagaki, J.-G. Delcros, J.-P. Moulinoux, Eur. J. Biochem. 243 (1997) 527–536.
[28] L. Hevlicek, J. Hanus, J. Vesely, S. Leclerc, L. Meijer, G. Shaw, M. Strnad, J. Med. Chem. 40 (1997) 408–412.
[29] W.F. De Azevedo, S. Leclerc, L. Meijer, L. Havlicek, M. Strnad, S.H. Kim, Eur. J. Biochem. 243 (1997) 518–526.
[30] U. Schulze-Gahmen, J. Brandsen, H.D. Jones, D.O. Morgan, L. Meijer, J. Vesely, S.H. Kim, Proteins 22 (1995) 378–391.
[31] P. Furet, J. Zimmermann, H.G. Capraro, T. Meyer, P. Imbach, J. Comput. Aid Mol. Des. 14 (2000) 403–409.
[32] R.K. Robins, J. Am. Chem. Soc. 79 (1957) 6407–6415.
[33] D.J. Brown, P.W. Ford, J. Chem. Soc. Sect. C (1967) 568–572.
[34] A. Toyota, N. Katagiri, C. Kaneko, Heterocycles 36 (1993) 1625–1630.
[35] J.Y. Lee, Y.K. Park, I.-S. So, H.-K. Chung, B.-S. Yang, S.J. Lee, H. Park, Y.S. Lee, Arch. Pharm. Pharm. Med. Chem. 334 (2001) 357–360.