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Synthesis, Crystal Structure Determination, and Biological Properties of the DNA-dependent Protein Kinase (DNA-PK) Inhibitor 3-Cyano-6-hydrazonomethyl-5-(4-pyridyl)pyrid-[1*H*]-2-one (OK-1035)

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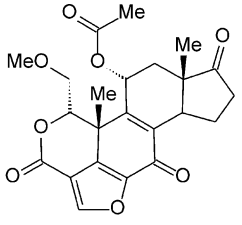
Abstract—The first reported synthesis of the DNA-PK inhibitor 3-cyano-6-hydrazonomethyl-5-(4-pyridyl)pyrid-[1*H*]-2-one (OK-1035) is described. The structure of OK-1035 was validated by X-ray crystallography. An IC_{50} value of 100 μM was determined for inhibition of DNA-PK, and this is approximately 12-fold higher than that reported previously.

Introduction

The nuclear serine/threonine protein kinase DNA-dependent protein kinase (DNA-PK) is a member of the phosphatidylinositol (PI) 3-kinase family, and consists of a catalytic subunit (DNA-PKcs), and a heterodimeric subunit (Ku).[1,2] DNA-PK recognises, and is activated, by DNA double-strand breaks (DSBs), and is a crucial component of the cellular DNA repair machinery. Importantly, DNA-PK is involved in the repair of DNA DSBs induced by ionising radiation and certain cancer chemotherapeutic agents, and also in the repair of DSBs induced during the physiological process of V(D)J recombination.[1] Inhibition of DNA-PK activity has been shown to potentiate the in vitro cytotoxicity of *X*-radiation[3,4] and a number of anticancer drugs.[5,6] Inhibitors may thus have clinical utility as radio- and chemo-potentiators in the treatment of cancer. In addition, a specific and potent inhibitor would be valuable in probing the role of DNA-PK in DNA repair, nuclear signal transduction and V(D)J recombination.[2]

The currently available DNA-PK inhibitors wortmannin (**1**) and LY294002 (**2**) lack kinase specificity. Both



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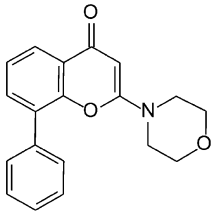

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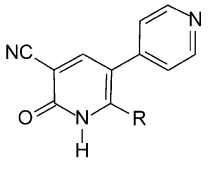

3; R = $CHNNH_2$
4; R = CH_3

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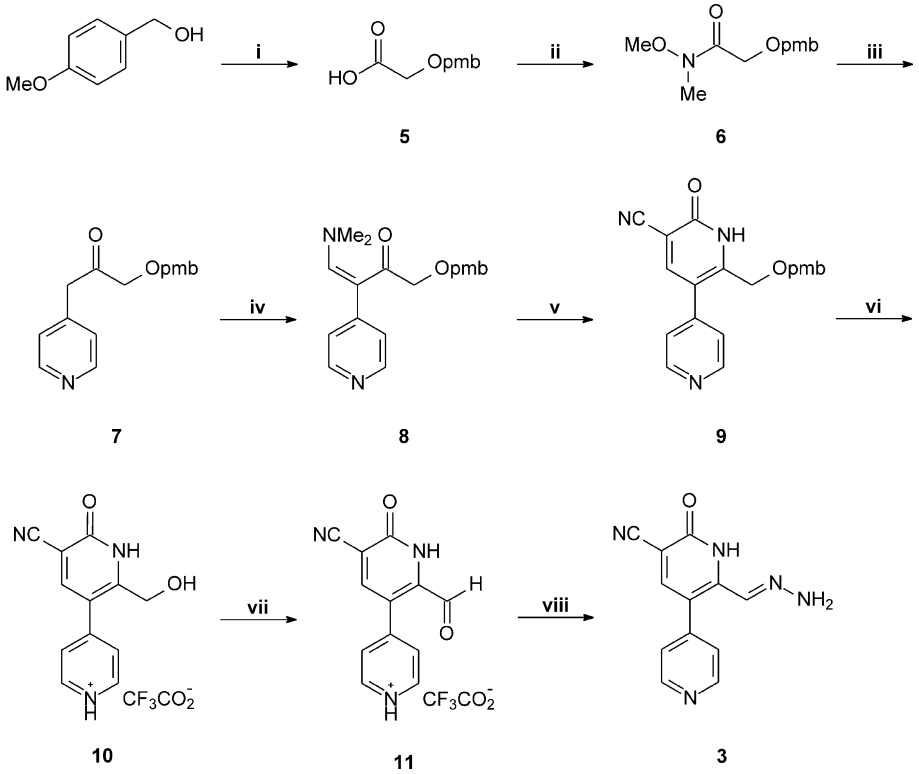
compounds are inhibitors of PI 3-kinase[4,7,8] and wortmannin also inhibits other members of the PI 3-kinase family including ATM and ATR.[9,10] OK-1035 (**3**) was identified through a screening programme, and is reportedly highly selective for DNA-PK compared with a number of other kinases, although activity against other PI 3-kinase family members was not determined.[11,12] As part of a research programme to develop potent and selective DNA-PK inhibitors, we wished to determine the activity of **3** against other members of the PI 3-kinase family, with a view to investigating further this potentially interesting compound. However, the synthesis of **3** does not appear to have been published, beyond a generic patent detailing the preparation of 4-pyridyl-2-pyridones as cardiotonic agents.[13] In this paper we report an efficient synthetic route to OK-1035, the structure of which was confirmed by crystal structure analysis, and the inhibitory activity of this compound against DNA-PK and related PI 3-kinases.

Chemical Synthesis

Our approach to the preparation of **3** was based on the reported synthesis of the structurally similar bipyridine phosphodiesterase inhibitor milrinone **4**,[14,15] suitably modified to enable introduction of the hydrazone function in the final step (Scheme 1). Initial attempts to prepare ketone **7** by direct acylation of 4-picoline with ethyl 2-(4′-methoxybenzyl)oxyethanoate and *n*-butyllithium were unsuccessful, resulting in a mixture of mono- and di-acylated products. This problem was avoided by utilising the method of Weinreb.[16] Thus, alkylation of 4-methoxybenzyl alcohol with bromoacetic acid afforded **5** in excellent yield, and this was converted smoothly into the Weinreb amide **6** on treatment with *N*,*O*-dimethylhydroxylamine in the presence of DCC. Metalation of 4-picoline with *n*-butyllithium, and reaction with **6**, gave the required 4-methoxybenzyl (pmb) protected α-hydroxyketone **7**. Conversion of **7** into enamide **8** was achieved with dimethylformamide dimethylacetal in DMF, and condensation of **8** with 2-cyanoacetamide afforded pyridone **9**.[17] Efforts to remove the pmb group of **9** by catalytic hydrogenation proved unsuccessful. However, deprotection of **9** occurred in high yield on treatment with ice-cold TFA, furnishing the required alcohol **10** as the trifluoroacetate salt. Oxidation of **10** with manganese dioxide gave the aldehyde **11**, and final treatment with an excess of hydrazine hydrate afforded OK-1035 **3** in good overall yield.[18]

Results and Discussion

The structure of **3** was confirmed by X-ray crystallography (Fig. 1).[19] The NH_2 group acts as a double hydrogen-bond donor: one N–H donates to the pyridyl N atom in an adjacent molecule, forming centrosymmetric dimers, while the other donates to a carbonyl oxygen atom, linking the dimers into sheets of molecules. By contrast, the pyridone NH group forms only a relatively weak intramolecular hydrogen bond with the nearer N atom of the hydrazone substituent, and the



Scheme 1. Synthesis of OK-1035 (**3**). Reagents and yields: (i) $BrCH_2CO_2H$, NaH, THF, reflux, 90–94%; (ii) MeONHMe·HCl, DCC, NEt_3, EtOAc, 25 °C, 75–80%; (iii) *n*-BuLi, THF, picoline, −78 → 0 °C, 75–76%; (iv) $Me_2NCH(OMe)_2$, DMF, 65 °C; (v) $N{\equiv}CCH_2CONH_2$, NaOMe, DMF, 95 °C, 45–50%; (vi) CF_3CO_2H, 0 °C, 98–100%; (vii) MnO_2, DMF, 25 °C; (viii) $N_2H_4{\cdot}H_2O$, MeOH, 25 °C, 25–30%. pmb = 4-methoxybenzyl.

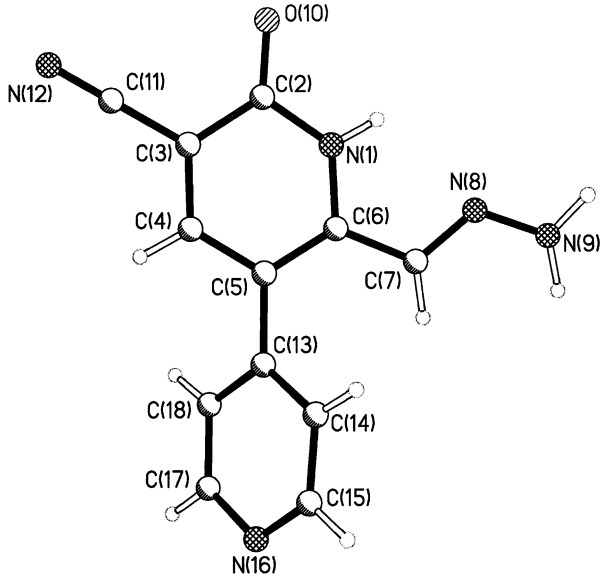


Figure 1. Molecular structure of OK-1035 (**3**).

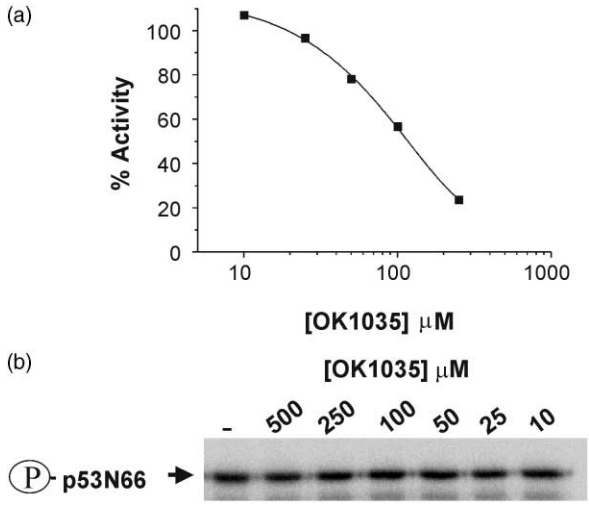


Figure 2. (a) OK-1035 inhibits DNA-PK in a peptide-based assay with an IC_{50} of 100 μM. (b) OK-1035 does not inhibit DNA-PK activity towards a p53 based protein substrate (p53N66), as judged by phosphorimage analysis following electrophoresis on a 12% SDS-polyacrylamide gel.

cyano group does not act as a hydrogen bond acceptor. Intramolecular geometry is unexceptional; all atoms except for the pyridyl group lie essentially in one plane, and the pyridyl ring is twisted by 45° relative to this plane.

The DNA-PK inhibitory activity of OK-1035 (**3**), and the two synthetic intermediates **9** and **10**, was determined as described previously[20] and the results are shown in Table 1. The activity of the benchmark inhibitor LY294002 (**2**) is included for comparative purposes. The lack of activity observed for **9** and **10** is consistent with previously reported structure–activity relationships, where a 6-hydrazonomethyl function was found to be essential for DNA-PK inhibition.[11] However, a marked difference was observed between the literature value for the potency of **3** ($IC_{50} = 8$ μM),[11] and the value obtained for the compound synthesised in our laboratory ($IC_{50} = 100$ μM; Fig. 2a). The reasons for this discrepancy are uncertain, and although we have utilised the same peptide substrate and ATP concentration as reported, may relate to some unknown difference in the DNA-PK assay. Interestingly, the authors observed a variation in the inhibitory activity of **3** with differing substrates, and while casein afforded an IC_{50} value of 40 μM, only weak inhibitory activity was observed at the highest concentrations of **3** (40 and 160 μM) when wild-type p53 was employed as a substrate. Similarly, when we used a wild-type p53 substrate we did not observe any significant inhibition in our assay at concentrations up to 500 μM as determined by phosphorimager scanning (Fig. 2b). Alternatively, it is possible that the DNA-PK inhibitor identified from the original screening programme, was structurally different from that reported as OK-1035.

Table 1. Inhibition of DNA-PK by selected compounds[a]

Compd number	DNA-PK inhibition IC_{50} (μM)[b]
2 (LY294002)	1.4
3 (OK-1035)	100
9	> 500
10	> 500

[a]DNA-PK was purified from HeLa cell nuclear extract as described previously[20] and assayed by the method of Lees-Miller[21] using peptide #15 (HPLC purified 97% pure) and a recombinant GST fusion protein containing the amino-terminal 66 amino-acids of wild-type human p53 (p53N66).

[b]Defined as the concentration of inhibitor required to reduce DNA-PK activity by 50% of control. Values are the means of three separate determinations.

The analytical and crystal structure data obtained for our sample of **3**, prove that the compound described in this paper is indeed 3-cyano-6-hydrazonomethyl-5-(4-pyridyl)pyrid-[1*H*]-2-one. Unfortunately, details of the synthesis and characterisation of the OK-1035 used in the original biological studies have not been published, and although we have requested an authentic sample of this material for comparative purposes, further quantities are no longer available. Finally, **3** was also found to be devoid of inhibitory activity against PI 3-kinase and ATM at a single concentration of 100 μM (data not shown). Thus, although in our hands **3** is a weak inhibitor of DNA-PK, the compound does perhaps exhibit some selectivity for this kinase over other members of the PI 3-kinase family. In summary, we have developed a viable synthetic route for the preparation of **3**, the structure of which has been unambiguously determined by X-ray crystallography. The DNA-PK inhibitory activity of **3** has been confirmed, albeit with a potency approximately 12-fold lower than that reported previously, and some evidence for selectivity for DNA-PK over related PI 3-kinases has been adduced.

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17. To a solution of bromoacetic acid (13.8 g, 0.10 mol) and 4-methoxybenzyl alcohol (13.8 g, 12.5 ml, 0.10 mol) in THF (180 mL) under nitrogen at 0 °C, was added sodium hydride (5.4 g, 0.24 mol) portionwise. The suspension was heated at reflux for 24 h and quenched with methanol. The mixture was concentrated in vacuo and the residue was partitioned between ether (70 mL) and water (70 mL). The ether layer was separated and washed with water (70 mL). The combined aqueous extracts were acidified to pH 4 with hydrochloric acid and the resulting solution was extracted with dichloromethane (3×150 mL). The combined extracts were dried (Na_2SO_4) and concentrated in vacuo. The oily residue was crystallised under cold ether. The white crystals were collected and dried to provide **5** (17.6 g, 0.09 mol, 94%), δ_H (200 MHz, $CDCl_3$) 3.79 (3H, s, OCH_3); 4.08 (2H, s, $ArCH_2$); 4.56 (2H, s, $COCH_2$); 6.87 (2H, d, Ar H-3,5); 7.26 (2H, d, Ar H-2,6). MS (EI) $m/z = 196$ (M^+). A solution of **5** (14.1 g, 72 mmol) in ethyl acetate (200 ml) was treated with *N,O*-dimethylhydroxylamine hydrochloride (7.20 g, 74 mmol), followed by 1,3-dicyclohexylcarbodiimide (15.7 g, 76 mmol). The stirred suspension was treated with triethylamine (8.05 g, 11.1 ml, 80 mmol) at 0 °C and the resulting mixture was maintained at room temperature for 16 h. The suspended solid was filtered off and washed with ethyl acetate. The filtrate was concentrated in vacuo to a volume of ca. 150 mL. The resulting solution was washed with ice-cold 1 M hydrochloric acid (2×100 mL), saturated aqueous $NaHCO_3$ (2×100 mL) and brine (100 mL), dried (Na_2SO_4) and concentrated in vacuo. The crude product was purified by medium pressure chromatography on silica (eluting with an ethyl acetate/petrol gradient) to give **6** (13.6 g, 57 mmol, 79%) as a colourless oil. Found C, 59.94; H, 7.17; N, 5.85%. $C_{12}H_{17}NO_4$ requires C, 60.24; H, 7.16; N, 5.85%; δ_H (200 MHz, DMSO-d_6) 3.16 (3H, s, NCH_3); 3.60 and 3.77 (6H, 2×s, 2×OCH_3); 4.21 (2H, s, $COCH_2$); 4.57 (2H, s, $ArCH_2$); 6.85 (2H, d, Ar H-3,5); 7.28 (2H, d, Ar H-2,6). MS (EI) m/z 239 (M+). To a solution of 4-picoline (10.8 g, 11.0 mL, 0.11 mol) in THF (120 mL) under nitrogen at −78 °C was added dropwise 2.5 M butyllithium in hexanes (40 mL, 0.10 mol). The solution was allowed to warm up to −10 °C, stirred at this temperature for 15 min and cooled to −78 °C. After addition of **6** (10.0 g, 41.8 mmol) in THF (40 ml), the mixture was warmed to 0 °C, stirred for 4 h and finally quenched with 2 M hydrochloric acid. The resulting solution was washed with ether (120 mL), cooled to 0 °C, and the pH was adjusted to 8 by addition of saturated aqueous $NaHCO_3$. The aqueous mixture was extracted with dichloromethane (3×250 mL) and the combined extracts were dried (Na_2SO_4) and concentrated in vacuo. The crude product was purified by medium pressure chromatography on silica (eluting with an ethyl acetate/petrol gradient) to give **7** (8.6 g, 31.7 mmol, 76%) as a yellow oil. δ_H (200 MHz, $CDCl_3$) 3.72 (2H, s, CH_2); 3.74 (3H, s, OCH_3); 4.01 (2H, s, CH_2); 4.44 (2H, s, CH_2); 6.82 (2H, d, $J = 8.4$ Hz, Ar H-3,5); 7.05 (2H, d, $J = 5.8$ Hz, pyridyl H-3,5); 7.18 (2H, d, $J = 8.4$ Hz, Ar H-2,6); 8.47 (2H, d, $J = 5.8$ Hz, pyridyl H-2,6). A solution of **7** (7.9 g, 29 mmol) in DMF (20 ml) was treated with dimethylformamide dimethylacetal (8.7 g, 9.7 mL, 73 mmol), under nitrogen, and heated at 70 °C for 5 h. The resulting mixture was concentrated in vacuo (4.3×10^{-3} Torr and < 50 °C) to give crude **8** that was used directly without further purification. A solution of **8** in DMF (25 mL) was added, with stirring under nitrogen, to sodium methoxide (3.4 g, 63 mmol) and 2-cyanoacetamide (2.7 g, 32 mmol) in DMF (20 mL). The mixture was heated at 95 °C for 3 h and concentrated in vacuo (3.8×10^{-3} Torr and < 50 °C). To the oily residue was added dichloromethane (150 mL) and water (50 mL), followed by 2 M hydrochloric acid until the pH of the aqueous solution was 7. The dichloromethane was separated and the aqueous layer was extracted with dichloromethane (4×120 mL). The combined extracts were dried (Na_2SO_4) and evaporated in vacuo. The crude product was purified by medium pressure chromatography on silica (eluting with a methanol/dichloromethane gradient). The solid product was recrystallised from ethyl acetate to give **9** (4.69 g, 13.5 mmol, 46%) as yellow crystals, mp 141–142 °C. Found: C, 69.13; H, 4.66; N, 12.01%. $C_{20}H_{17}N_3O_3$ requires C, 69.15; H, 4.93; N, 12.10%; δ_H (200 MHz., DMSO-d_6) 3.84 (3H, s, OCH_3); 4.40 (2H, s, OCH_2Ar); 4.49 (2H, s, CH_2OCH_2Ar); 6.97 (2H, d, Ar H-3,5); 7.28 (2H, d, Ar H-2,6); 7.50 (2H, d, pyridyl H-3,5); 8.38 (1H, s, pyridone H-4); 8.69 (2H, d, pyridyl H-2,6); 13.06 (1H, s, NH). MS (EI) m/z 347 (M+).
18. A solution of **9** (1.00 g, 2.9 mmol) in trifluoroacetic acid (5 mL) was stirred for 2 h under nitrogen at 0 °C. After concentration of the resulting solution in vacuo, the crude product was triturated with anhydrous ethanol. The solid product was collected by filtration and dried to give **10** (0.97 g, 2.8 mmol, 98%) as a beige powder, mp ~140 °C (decomp). IR (KBr) ν_{max} 3225; 3065; 2214 (cyano); 1628 (pyridone); 1497; 1566; 1127 cm^{-1}; δ_H (200 MHz., DMSO-d_6) 4.55 (2H, s, CH_2); 7.98 (2H, d, $J = 5.2$ Hz., pyridyl H-3,5); 8.48 (1H, s, pyridone H-4); 8.94 (2H, d, $J = 5.2$ Hz, pyridyl H-2,6); 12.79 (1H, s, NH). A solution of **10** (0.80 g, 2.5 mmol) in DMF (10 mL) was stirred with activated manganese dioxide (3.0 g) for 2 h under nitrogen. The resulting mixture was filtered three times through Celite, washing well with methanol each time, and the combined filtrates were concentrated in vacuo to provide **11** as a crude oil. A solution of hydrazine hydrate (3.09 g, 3.0 mL, 52 mmol) in methanol (10 mL) was added to a suspension of

crude **11** (assumed 2.5 mmol) in methanol (10 mL), and the resulting mixture was stirred for 16 h and concentrated in vacuo. The residual solid product was washed with ether (3×30 mL) and purified by medium pressure chromatography on silica (eluting with a methanol/dichloromethane gradient), to give **3** (0.16 g, 0.67 mmol, 26%) as a yellow powder, mp > 240 °C (decomp.). Found: C, 59.31; H, 3.56; N, 28.61%. $C_{12}H_9N_5O{\cdot}0.2\ H_2O$ requires C, 59.35; H, 3.90; N, 28.84%; IR (KBr) ν_{max}: 3360; 3286; 3036; 2217 (cyano); 1656 (pyridone); 1595; 1566; 1523 (hydrazono); 1273; 1224; 835; 791; 601 cm^{-1}; δ_H (200 MHz, DMSO-d_6) 7.43 (1H, s, C*H*NNH_2); 7.50 (2H, d, $J = 5.7$ Hz, pyridyl H-3,5); 8.23 (1H, s, pyridone H-4); 8.67 (2H, d, NH_2); 8.74 (2H, d, $J = 5.7$ Hz, pyridyl H-2,6); 11.48 (1H, s, NH); δ_C (50 MHz, DMSO-d_6) 100.0; 114.0; 116.6; 123.2; 124.5; 142.9; 145.3; 149.8; 149.9; 159.0; MS (EI) m/z 239 (M^+); 223 ($M^+ - NH_2$, 31%); 211 ($M^+ - N_2$, 97%); UV (λ_{max}) 301, 398 nm.

19. The structure of **3** was determined from data collected at 160 K on a Bruker AXS SMART CCD diffractometer with Mo-K_α radiation ($\lambda = 0.71073$ Å). Crystal data: $C_{12}H_9N_5O$, $M_r = 239.2$, monoclinic, space group $P2_1/c$, $a = 10.3494(7)$, $b = 11.7648(8)$, $c = 10.6187(7)$Å, $\beta = 118.784(2)°$, $V = 1133.17(13)$ Å^3, $Z = 4$; $R(F;F^2 > 2\sigma) = 0.038$, $R_w(F^2$; all data) = 0.109, $S = 1.05$ for 2686 unique data and 199 parameters, final difference synthesis within ±0.24 e Å^{-3}. Crystallographic data (excluding structure factors) have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 161304. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge CB2 1EZ, UK [fax: +44 (1)1223 336033 or e-mail: deposit@ccdc.cam.ac.uk].

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