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3-Acetoxy-2,2'-Bi-1H-indol as A Novel Inhibitor of ATP Binding to DnaA, the Protein Initiating Chromosomal Replication in *Escherichia coli*

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Abstract:

3-Acetoxy-2,2'-bi-1H-indol which is derived from indigo was found to be a novel and potent inhibitor of ATP binding (IC_{50}=0.04mM) to DnaA protein, a key protein in the initiation of chromosomal DNA replication in *Escherichia coli*. Competitive experiments indicated the inhibition to be non-competitive with ATP.

Introduction The initiation of DNA replication of *E. coli* begins at a unique site (*oriC*), and requires *oriC*-binding DnaA protein. DnaA protein is activated by binding with ATP to open the duplex in the *oriC* region, a necessary stage for subsequent replication. DnaA protein has also high binding affinity for ADP, but the ADP-bound DnaA protein is inactive in initiation.[1] Thus, binding of ATP to DnaA protein is thought to be a crucial step in the initiation of DNA replication. Phospholipids (*e.g.* cardiolipin), a major component of biomembrane, dissociate both ATP and ADP-bound DnaA protein at a relatively high temperature.[2,3] To investigate the biological significance of ATP binding to DnaA protein, selective inhibitors which mediate this process are highly desired, but have not been so far reported. Such inhibitors may be also therapeutically useful. We report here the first inhibitors for ATP binding to DnaA protein *in vitro* .

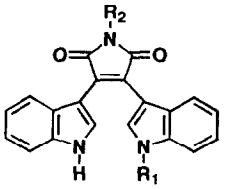
Bisindolylmaleimide derivatives (**1**) have been estimated as inhibitors of ATP binding to protein kinases such as protein kinase C, tyrosine kinase, and so on, and are regarded as a basic skeleton for the interaction with ATP binding pocket of protein kinases.[4] Based on the assumption that the ATP binding pocket of DnaA protein may resemble those of protein kinases, we investigated the inhibition of ATP binding to DnaA protein with **1a-c** (Table 1). Among the derivatives (**1a-c**), the least substituted **1a** showed the most potent inhibition activity. As no information was available for

inhibitors of ATP binding of DnaA, we further sought more potent inhibitors considering the following facts: 1) two aromatic rings attached to maleimide seem to be necessary for high affinity with ATP binding pocket of kinases,[5] and 2) the fact that bulky substituent on the maleimide diminished inhibitory activity (Table 1). Thus, 2,2'-bi-1H-indol derivatives such as shown in Scheme 1 were designed for further investigation from structural simplification of the bisindolylmaleimide skeleton of **1**. Acetyl- (**2** and **4**) and ether derivatives (**3**) were used to test basic structure-activity relationship in inhibition of ATP binding of DnaA.

Table 1 Preliminary Data of Inhibition of [α-^{32}P]ATP binding to DnaA Protein

	Compounds		IC_{50} (mM)[a]
	R_1	R_2	
1a	H	H	1
1b	$(CH_2)_3NMe_2$	H	3
1c	H	CH_2Ph	>10

a) The concentration needed for 50% inhibition of ATP binding to DnaA. Experiments were performed as described in the text.



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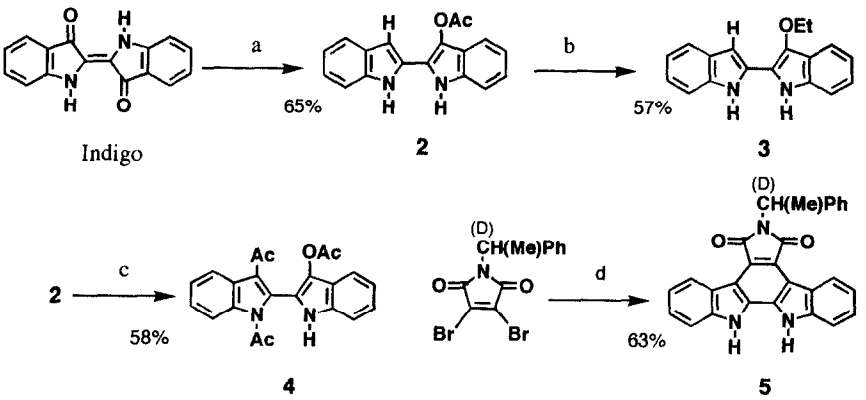
Materials and Methods [2,2'-Bi-1H-indol]-3-ol acetate (**2**) was synthesized from indigo by the reaction with hydrazine[6] under argon atmosphere followed by treatment with acetic anhydride (Scheme 1).[7] Without acetylation, the reduced product could not be isolated and only complexed mixture was obtained. The ether derivative (**3**) was derived from **2** to investigate the effect of the carbonyl group of **2**. The acetate (**2**) was further acetylated with $ZnCl_2$ in acetic anhydride to produce triacetylated compound **4**.[7] Bisindolylmaleimide (**5**) was synthesized from dibromomaleimide derivative by the similar method as described in the literature,[8] and used to investigate the influence of bulky maleimide.

Scheme 1[7]



a) i) NH_2NH_2, NaOH, EtOH, 90°C, 4h; ii) Ac_2O, b) (nBu)$_4$OH, EtI, THF, rt, 1h, c) $ZnCl_2$, Ac_2O, rt, 30 min, d) i) PhMgBr, indol, THF, reflux, 3d, ii) DDQ, TsOH, benzene, reflux, 1h

Inhibition of ATP binding to DnaA ATP-binding to DnaA protein purified from *E. coli*[3] was assayed as follows.[1] Various amounts of the compounds were added to a solution of DnaA protein followed by incubation with [α-^{32}P]ATP at 0°C for 15 min in buffer, then the solution was filtered through a nitrocellulose membrane. Radioactivity retained on the filter was measured in a liquid scintillation counter. Fig. 1 shows dose-dependent inhibition curves, and concentrations at 50% inhibition (IC_{50}) are summarized in Table 2. The least substituted 3-acetoxy-2,2'-bi-1H-indol (**2**) exhibited the most potent inhibition (IC_{50}=0.04 mM). Interestingly, indigo itself inhibited ATP binding (IC_{50}=0.2 mM). In the control experiments with indol and adenosine, no inhibition was observed, thereby indicating 2,2'-bi-1H-indol skeleton may be an essential structural component for inhibitors.

Fig 1. Dose-Dependent Inhibition Curves of ATP binding to DnaA Protein

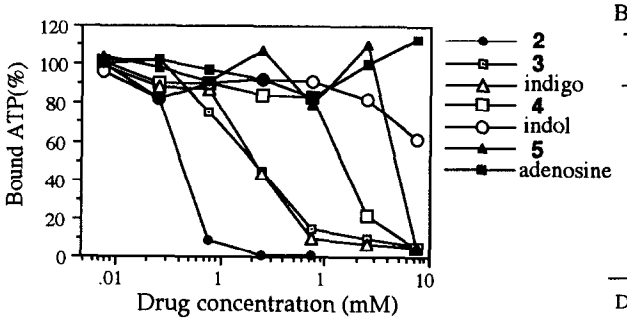


Table 2 IC_{50} of Several Compounds in [α-^{32}P]ATP Binding to DnaA Protein

Compounds	IC_{50} (mM)
2	0.04
3	0.2
4	1.5
5	5
indigo	0.2
indol	>10
adenosine	>10

Data taken from Fig. 2.

[α-^{32}P]ATP binding to DnaA protein was assayed by membrane binding at 0°C. DnaA protein and compounds were mixed prior to the incubation with [α-^{32}P]ATP.

Inhibition Mode Effects of an excess of ATP on the inhibition of [α-^{32}P]ATP binding to DnaA protein were examined using the inhibitor **2** (Fig. 2), and almost the same inhibition curves were obtained, regardless of the amount of ATP present. These results indicate that the mode of inhibition is non-competitive to ATP. In experiments in which DnaA protein was incubated with [α-^{32}P]ATP prior to the addition of **2** at 0°C, probably a tight ATP-DnaA complex formed, and no inhibition was observed (Fig. 3). It is previously reported that phospholipids (cardiolipin) which inhibit ATP binding to DnaA protein cannot dissociate previously formed ATP-DnaA complex under the same condition at 0°C.[2] Higher temperature at 38°C was needed for cardiolipin to dissociate previously formed ATP-DnaA complex, probably accomanying denaturation of DnaA protein. It is not clear at this stage whether the inhibior **2** interacts with the ATP-binding site or with an allosteric site.

Further studies on effects of **2** on DNA replication and development of even more potent inhibitors are ongoing.

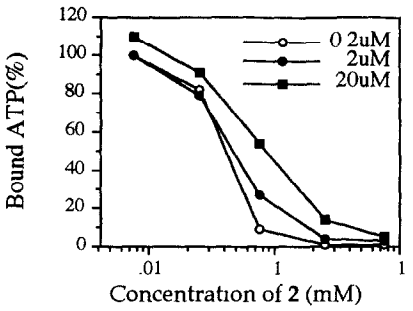


Fig. 2. Effect of An Excess ATP on the Inhibition.

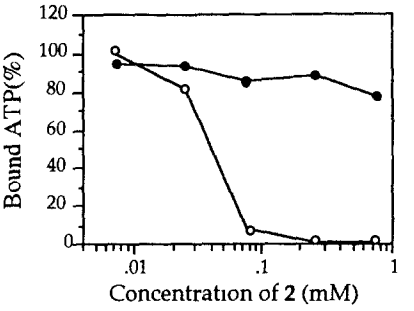


Fig. 3. Effect of Order of Addition on Inhibition

—o— DnaA protein and **2** were incubated prior to the incubation with ATP

—●— DnaA protein and ATP were incubated first follwed by the addition of **2**

Acknowledgments

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References and Notes

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7) Satisfactory spectral and analytical data were obtained for all compounds listed here. Selected spectral and physical data are as follows. ^{1}H-NMR (δ ppm in $CDCl_3$): **2** (2.508, s, 3H, CH_3), (6.694, dd, 1H, *J*=2.5, 1.4 Hz, 2'H), **3** (1.431, t, 3H, *J*=7.0 Hz, CH_3), (4.340, q, 2H, *J*=7.0 Hz, CH_2), (6.542, d, 1H, *J*=1.6 Hz, 2'H), **4** (1.978, 2.098, 2.237, s, 3Hx3, CH_3), **5** (2.100, d, *J*=8.8 Hz, 3H, CH_3), (5.75, q, 1H, *J*=8.8 Hz, CH-Ph). Melting point: **2** (180-182°C), **3** (168-170°C), **4** (132-136°C), **5** (295-300°C). The acetylated nitrogen of **4** may be interchangeable.

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