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Structure–activity relationship studies of the chromosome segregation inhibitor, Incentrom A

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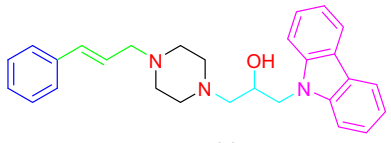
ABSTRACT

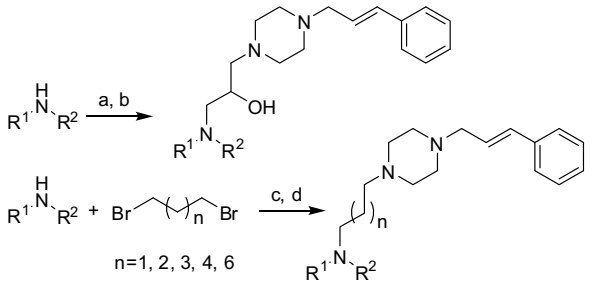
A series of Incentrom A analogs that inhibit the chromosome segregation process in yeast were synthesized and tested for their effects on chromosome stability and cell proliferation. Pharmacophore and structure–activity relationship of Incentrom A for the anti-yeast activity were established.

The process of chromosome segregation is crucial for the conservation of normal chromosome number in all eukaryotes. Accurate chromosome segregation requires specialized chromosomal structures called the centromere (DNA region) and the kinetochore (protein assembly). The centromeres of the experimental model yeast *Saccharomyces cerevisiae* contain specific and absolutely essential DNA sequences for faithful chromosome segregation, whereas those of higher organisms have extensive amounts of repetitive sequences.[1,2] Besides, the centromeres of the pathogenic yeast *Candida glabrata*, the second most common fungal pathogen in humans, are closely related to the *S. cerevisiae* centromeres.[3] Thus, small molecules that perturb centromere functions in *S. cerevisiae* could be valuable in identifying yeast-specific growth inhibitors to lay the basis for the development of novel antifungal drugs.[4] Incentrom A is one such compound.[4] To probe the molecular target of Incentrom A and eventually develop agents which selectively block the growth of fungi, we studied the pharmacophore and structure–activity relationship (SAR) of Incentrom A and obtained a compound for photo-affinity labeling.[5]

Incentrom A (**1**) has several pharmacophoric units as the two aromatic moieties are attached to the piperazine ring through alkyl chains. To establish the structure–activity relationship for Incentrom A, we synthesized a series of Incentrom A derivatives and measured a minimum inhibitory concentration (MIC) for each compound.[6]



Incentrom A (1)



Scheme 1. Reagents and conditions: (a) epibromohydrin, Cs_2CO_3, DMF, 60 °C, 15 h; (b) *trans*-1-cinnamylpiperazine, THF, EtOH, 60 °C, 1 day. (c) TBAB, benzene, 50% aq. NaOH, rt, 1 day; (d) *trans*-1-cinnamylpiperazine, TBAB, CH_2Cl_2, 50% aq. NaOH, rt, 1 day.

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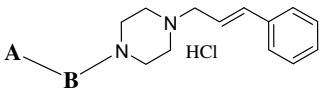
Table 1
Yeast growth inhibitory activities for compounds **1–21**



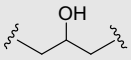
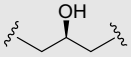
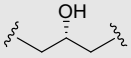
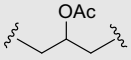
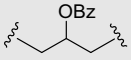
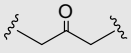
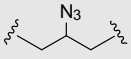
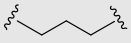
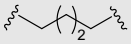
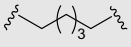
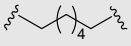
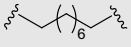
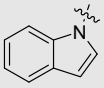
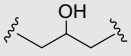
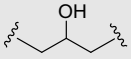
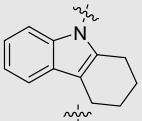
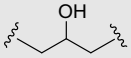
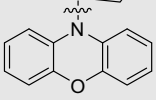
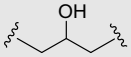
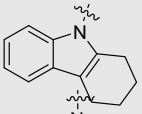
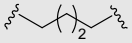
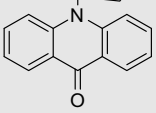
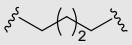
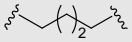
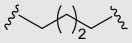
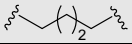
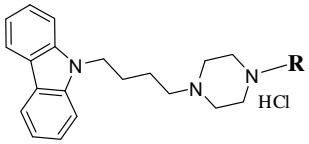
Compound	A	B	MIC (μM)
Incentrom A(1)	Carbazole		15
2	Carbazole		14–15
3	Carbazole		15–16
4	Carbazole		18–19
5	Carbazole		>45
6	Carbazole		>45
7	Carbazole		>45
8	Carbazole		12–13
9	Carbazole		5–6
10	Carbazole		6
11	Carbazole		7–8
12	Carbazole		44–45
13			>45
14			45
15			25–26
16			7–9

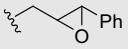
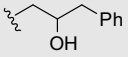
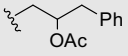
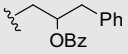
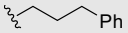
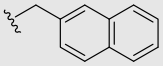
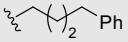
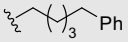
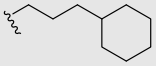
Table 1 (*continued*)

Compound	A	B	MIC (μM)
17			10–11
18			>45
19	3-Nitrocarbazole		5–6
20	3-Acetamidocarbazole		>45
21	3-Benzamidocarbazole		>45

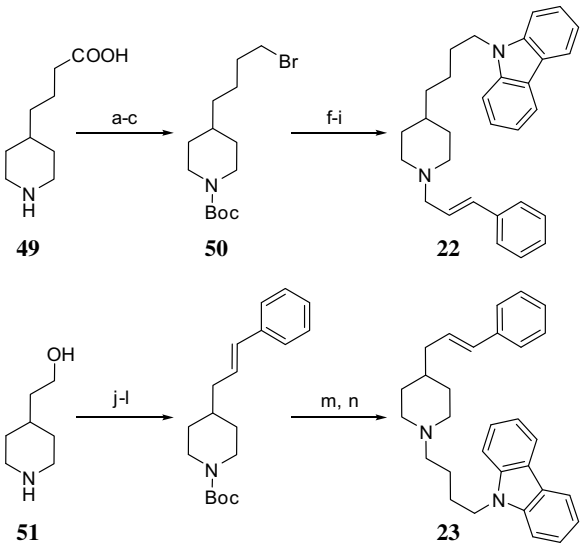
First, we examined the effects of functional groups and chain length between piperazine and carbazole nitrogens on the activity as well

Table 2
Yeast growth inhibitory activities for compounds **26–36**

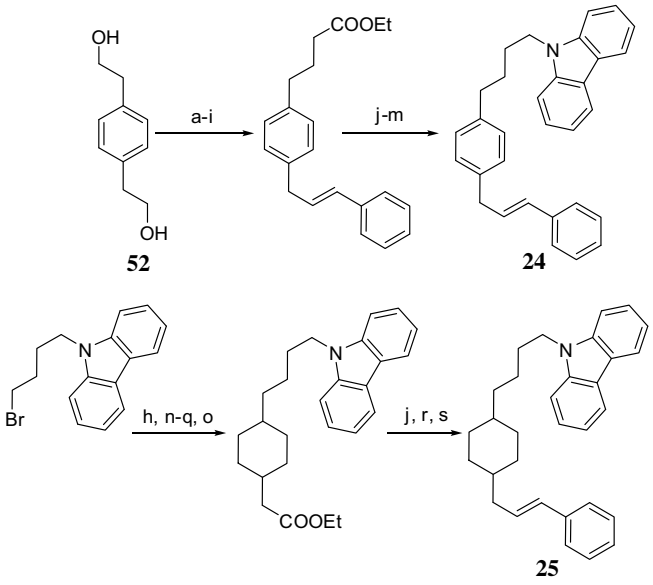


Compound	R	MIC(μM)
26		7–9
27		12–14
28		18–20
29		>45
30[a]		20
31		30
32[a]		>45
33		9–11
34		5–6
35		4–5
36		5–6

[a] 2-Hydroxypropyl chain in place of butyl chain between carbazole and piperazine rings.



Scheme 2. Reagents and conditions: (a) (trimethylsilyl)diazomethane, benzene, MeOH, rt, 1 h; (b) di-*tert*-butyl dicarbonate, DIPEA, DMAP, MeOH, rt, 16 h; (c) DIBAL, THF, 0 °C to rt, 100 min; (d) MsCl, Et_3N, CH_2Cl_2, 0 °C, 30 min; (e) LiBr, acetone, reflux, 12 h; (f) carbazole, Cs_2CO_3, DMF, 70 °C, 1 day; (g) TFA, CH_2Cl_2, rt, 45 min; (h) *trans*-cinnamaldehyde, AcOH, MeOH, rt, 1 h; (i) $NaBH(OAc)_3$, rt, 1 day. (j) di-*tert*-butyl dicarbonate, Et_3N, CH_2Cl_2, rt, 16 h; (k) oxalyl chloride, DMSO, Et_3N, CH_2Cl_2, −78 °C to rt, 5 h; (l) diethyl bezylphosphonate, NaH, DMF, 0 °C to rt, 17 h; (m) TFA, CH_2Cl_2, rt, 1 h; (n) *N*-(4-bromobutyl)carbazole, TBAB, CH_2Cl_2, 50% aq. NaOH, rt, 1 day.



Scheme 3. Reagents and conditions: (a) TBSCl, NaH, THF, 0 °C to reflux, 3 days; (b) MsCl, Et_3N, CH_2Cl_2, 0 °C, 20 min; (c) diethylmalonate, NaH, TBAI, THF, 0 °C to rt, 1 day; (d) NaCl, DMF, reflux, 38 h; (e) TBAF, THF, rt, 90 min; (f) MsCl, Et_3N, CH_2Cl_2, 0 °C, 20 min; (g) LiBr, acetone, reflux, 9 h; (h) PPh_3, CH_3CN, reflux, 1 day; (i) benzaldehyde, *t*-BuOH, benzene, 70 °C, 10 h; (j) $LiAlH_4$, THF, 0 °C, 20 min; (k) MsCl, Et_3N, CH_2Cl_2, 0 °C, 20 min; (l) LiBr, Acetone, reflux, 4 h; (m) carbazole, Cs_2CO_3, DMF, 80 °C, 14 h; (n) 1,4-cyclohexanedione monoethylene acetal, *n*-BuLi, THF, −78 °C to rt; (o) Pd/C, H_2(1 atm), ethylacetate, 7 h, rt; (p) 1 N HCl, acetone, 50 °C, 1 h; (q) (carbethoxymethylene) triphenylphosphorane, benzene, reflux, 3 days; (r) PCC, CH_2Cl_2, rt, 1 h; (s) diethyl benzylphosphonate, NaH, DMF, 0 °C to rt, 19 h.

as the importance of the carbazole ring in **1**. These analogs were readily synthesized from amine, monosubstituted piperazine and dibromoalkanes (or epibromohydrin) (Scheme 1).[7] The cell proliferation inhibitory activities of compounds **1**–**21** are summarized in Table 1.

Compared to racemic Incentrom A (**1**), separately prepared enantiomers (**2**, **3**) did not show any changes in activity, nor did the protection of the hydroxyl group (**4**). However, a group bigger than hydroxyl (**5**, **7**) or the sp^2 center in the chain (**6**) was not tolerable for activity. Actually, the hydroxyl group was not necessary because the compound without hydroxyl group improved the activity (**8**). Moreover, the butyl and pentyl chains showed better activities than other chain lengths (**9–12**), suggesting that the optimal moiety between carbazole and piperazine rings was a 4-carbon chain with no substituent in the middle.

When indole ring was introduced in place of carbazole (**13**) or the rigid carbazole ring was dismantled (**14**), the inhibitory activity was completely lost. On the other hand, ring expansion (**16**) or partially hydrogenated carbazole was tolerated (**15, 17**), whereas an introduction of the electron-withdrawing spacer was not (**18**). Of the substituted carbazoles (**19–21**), only the parent 3-nitrocarbazole improved the inhibitory activity.

Based on these results, we kept carbazole, fixed the C4 chain length between carbazole and piperazine rings, and explored the other parts of Incentrom A to place a photo-affinity tag.

We prepared piperidine analogs of Incentrom A to test the importance of two nitrogen atoms. Compound **22** was prepared from 4-piperidinyl butanoic acid (**49**) by converting **49** into the corresponding bromide (**50**) and by attaching carbazole and cinnamyl groups to **49**. The other piperidine analog **23** was prepared from 4-piperidinyl ethanol (**51**) as shown in Scheme 2. All carbon analogs (**24**, **25)** were prepared as shown in Scheme 3. The selective protection of **52** followed by two carbon extension on one side using malonate ester synthesis produced a butyl chain for attachment of a carbazole unit and the other ethyl alcohol was extended to the cinnamyl group through Wittig olefination reaction. Final attachment of carbazole produced **24**. Compound **25** was prepared from cyclohexanedione monoethyleneketal. A butylcarbazole unit was introduced through Wittig olefination followed by hydrogenation of the olefin product. After deprotection of the ketal, two-carbon extension was achieved using Wittig olefination reductions, thus leading to the *trans* isomer as the major form of disubstituted cyclohexane.

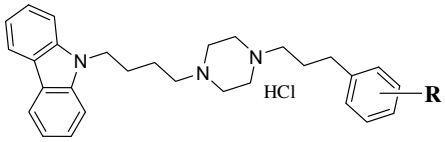
Finally, the cinnamyl unit was introduced in the same manner as in the synthesis of **24**.

The activities of these compounds were unusual: Both **22** and **23** exhibited undiminished inhibitory activities (MIC of 11–13 and 4–5 μM, respectively), whereas neither **24** nor **25** showed inhibitory activity. These results manifested that the presence of nitrogen in the molecule was crucial for the inhibitory activity. However, the position of the nitrogen atom was not critical. The nitrogen atom could play a role either in establishing electrostatic interaction with target proteins or in retaining the solubility in aqueous solution or both.

Next, we varied the cinnamyl group. An epoxide analog **26** was prepared similarly to the preparation of **9** using epoxycinnamyl bromide rather than cinnamyl bromide. Hydrogenolysis of **26** produced **27** and its ester analogs **28** and **29** were prepared from **27**. Hydrogenation of the double bond of **9** produced **30**. Conformationally restricted naphthyl compound (**31**), compounds with varying chain length (compounds **32-35**), and fully saturated cyclohexyl compound (**36**) were also prepared, and their activities are summarized in Table 2. An introduction of epoxide in place of olefin (**26**) slightly improved the activity, and the opening of epoxide to alcohol (**27**) retained the activity. The hydroxyl group offered a site for attaching other functional groups because the acetate form **28** was still active. However, the activity was lost when a larger group was attached (**29**). The saturation of the double bond of cinnamyl group (**30**) slightly reduced the activity.

Conformationally restricted naphthyl group (**31**) significantly lowered the activity. The benzyl group (**32**) completely shut off the activity. The extension of the chain length (**33**–**35**) gradually restored the activity. These results indicated that a flexible alkyl chain could help folding the compound around the parent piperazine ring. Nonetheless, the presence of benzene ring was not crucial as the cyclohexyl ring (**36**) showed better activity than **9**. Thus, these Incentrom A analogs could pose a sizable hydrophobic binding motif. Further optimization of the chain length and attachment of larger hydrophobic groups could help provide insights into the size of the hydrophobic binding pocket.

Table 3
Yeast growth inhibitory activities for compounds **37–48**



Compound	R	MIC (μM)
37	*p*-OH	28–29
38	*p*-OMe	8–9
39	*p*-OBn	>45
40	*p*-OC_6H_{13}	>45
41	*p*-OC_8H_{17}	>45
42	*m*-OH[a]	27–28
43	*m*-OMe[a]	12–13
44	*m*-OMe[a]	12–14
45	*p*-NH_2	16–17
46	*p*-NH_2[a]	15–16
47	*p*-NMe_2[a]	42–44
48	*p*-N_3[a]	5

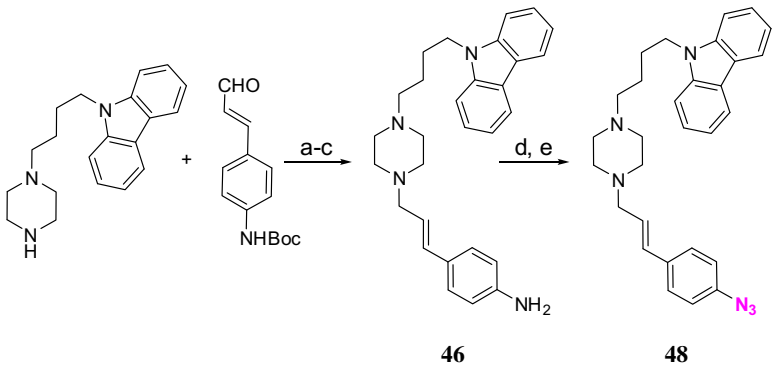
[a] These substituents were attached on the cinnamyl group.

Finally, to establish a proper site for attaching affinity tag, we substituted the phenyl ring of **9.** The results are summarized in Table 3. The polar phenolic group at the *para* and *meta* positions was well tolerated (**37**, **42**). The activity was improved when phenolic OH was replaced with OMe. The methoxy substitution at any position of the phenyl ring did not change the activity (**38**, **43**, **44**).

When the *para*-methoxy group was extended further with a longer alkyl chain or benzyl group, the activity was completely lost. These results indicated that this binding site could tolerate hydrophobic groups but only in a limited space. On the other hand, the activity got improved (**45**, **46**) when phenolic OH was replaced with an amine functional group. Although the dimethyl substitution of the amine quenched the activity, an introduction of an azide functional group showed an excellent activity (**48**). Since the azide group is a good candidate for photo-affinity labeling, compound **48** could serve as the probe for identifying the target protein of Incentrom A.

Compounds **46** and **48** were readily prepared from *p*-aminocinnamaldehyde in a few steps (Scheme 4). Reductive amination provided **46** and diazotization followed by azide replacement produced **48**. This synthetic scheme provided enough material for the photo-affinity labeling study and allowed synthesis of deuterated compound for mass spectrometric identification of target proteins.[8] Both in vitro and in vivo identification of target proteins are under way. In addition, the chromosome missegregation activities of Incentrom A (**1**) and its important analogs, **9** and **48**, are shown in Fig. 1.[9] As evidenced by a dramatic increase in red colonies, these compounds significantly induced a tester chromosome loss at much lower concentration than Incentrom A.

In summary, we established the pharmacophore of Incentrom A: The carbazole unit with four carbon tether was optimal for the inhibitory activity (**9**), and at least one nitrogen atom of the piperazine ring with a long hydrophobic chain was essential. We also identified a phenylazide containing compound **48** as a



Scheme 4. Reagents and conditions: (a) AcOH, MeOH, rt, 2 h; (b) $NaBH(OAc)_3$, rt, 1 day; (c) TFA, CH_2Cl_2, rt, 45 min; (d) conc. HCl, $NaNO_2$, 0 °C, 15 min; (e) NaN_3, 0 °C, 20 min.

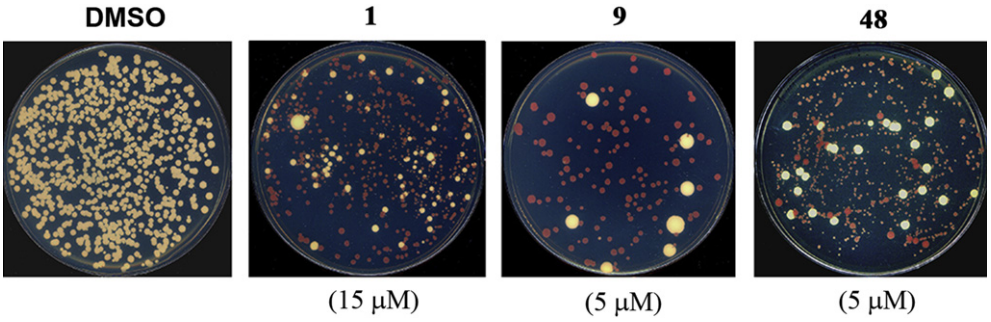


Figure 1. Chromosome missegregation activities of Incentrom A analogs.

photo-affinity probe to isolate target proteins in vivo. The present structure–activity relationship study provided further insights into the rational design of Incentrom A analogs.

Acknowledgment

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6. The MIC was defined as the lowest drug concentration at which 95–100% inhibition of yeast viability (YPH278 strain) was observed in comparison to the DMSO control. The 10 mM stock solution of each compound was prepared in DMSO. Yeast cells ($\sim 6.6 \times 10^5$ cells/mL) were treated with DMSO or Incentrom A analogs and grown for 24 h at 30 °C. Appropriate dilutions of each culture were plated on YPD medium. Yeast colonies were counted to calculate% reduction in yeast viability. The number of viable cells obtained from chemical-treated culture was normalized to the one from DMSO culture. Each MIC represents a mean or a range of values from 2–4 independent experiments.
7. (a) Bombrun, A.; Gerber, P.; Casi, G.; Terradillos, O.; Antonsson, B.; Halazy, S. *J. Med. Chem.* **2003**, *46*, 4365; (b) Li, X.; Mintz, E. A.; Bu, X. R.; Xehnder, O.; Bosshard, C.; Günter, P. *Tetrahedron* **2000**, *56*, 5785.
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9. The chromosome missegregation assay: A single colony of yeast strain YPH278 carrying a tester minichromosome was grown in 5 mL of selective medium to log phase ($OD_{660} = 2$). Subsequently $\sim$10 generations of non-selective growth were initiated by inoculating 20 mL of YPD with 20 μL of the selectively grown culture and treating with various compounds or DMSO. During the non-selective growth period, yeast cells that lost a tester chromosome produce red-colored colonies.