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Alexandra Botz, Yves Chenavier, Jacques Pécaut, Pascale Delangle, Christelle Gateau

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Graphical Abstract

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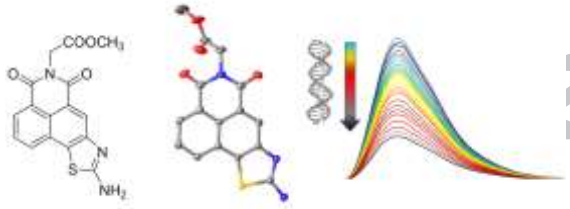

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Thiazonaphthalimide Derivatives: Synthesis and Interaction with DNA

Alexandra Botz, Yves Chenavier, Jacques Pécaut, Pascale Delangle and Christelle Gateau*

Univ. Grenoble Alpes, CEA, CNRS, INAC-SyMMES, 38000 Grenoble, France.

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ABSTRACT

The synthesis of several thiazonaphthalimide derivatives is described. The exclusive formation of angular rather than linear isomers was unequivocally demonstrated by NMR and single crystal X-ray diffraction. Their photophysical properties and ability to bind calf-Thymus DNA with affinities in the range of 10^4 makes them interesting candidates to probe DNA by fluorescence.

* Corresponding author. Tel : +33 4 38 78 60 41
e-mail address: christelle.gateau@cea.fr

The design of polyaromatic molecules able to bind to DNA is of significant importance for the development of anticancer and fluorescent imaging agents. Among them, 1,8-naphthalimide derivatives have received significant attention and have been extensively investigated for their potent antitumor activity.[1,2,3,4] Moreover their photophysical properties make them interesting candidates to detect DNA by fluorescence.[4]

Due to its tricyclic planar ring structure, 1,8-naphthalimide (Fig. 1) interacts with DNA and its anticancer activity was discovered 20 years ago.[6] A large variety of 1,8-naphthalimide derivatives has been designed and synthesized in order to improve the interaction with DNA and has been studied as anticancer and fluorescent imaging agents (Fig. 1).[2,3,4] Synthetic strategies such as functionalization of the imide nitrogen atom, substitution at the 3 and 4 positions and ring expansion, have provided access to a wide range of 1,8-naphthalimide derivatives with various chemical and photophysical properties. The position and nature of the substituents significantly influence the behavior of these derivatives in terms of DNA binding and fluorescence properties.[2,3] Interestingly, the aromatic fused derivative azonafide (Fig. 1) which contains an anthracene unit, displays a larger affinity for DNA than its parent compound amonafide with a naphthalene unit.[6] This result can be explained by the increased planar surface of the chromophore, thereby increasing the π-stacking interactions between the aromatic rings and the base pairs of DNA.

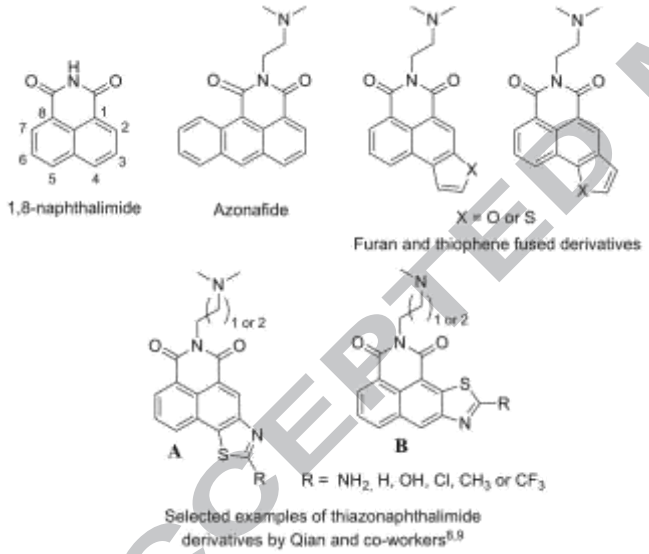


Figure 1. 1,8-naphthalimide and selected derivatives

The heteroaromatic fused derivatives with furan or thiophene developed by Brañas and co-workers demonstrate increased antitumor activity.[7] In fact, heteroaromatic rings likely contribute to additional interactions such as Van der Waals forces.[7] Qian and co-workers have described a novel family of naphthalimide derivatives, thiazonaphthalimides, in which the naphthalene ring has been fused with a thiazole moiety (Fig. 1 – **A** and **B**).[8] According to their study, the angular isomer **A** and the linear isomer **B** of the aminothiazole derivative (R = NH_2) were both obtained and interact with calf-Thymus DNA (ct-DNA) with an affinity in the range of $10^{4.5}$ M^{-1}. Following these promising early results, new angular and linear thiazonaphthalimide derivatives were reported by the same team and binding constants to DNA in the range of 10^{5-6} M^{-1} were achieved.[9]

The photophysical and DNA-binding properties of thiazonaphthalimide derivatives make them ideal candidates for the design of luminescent DNA probes. In particular their insertion into lanthanide complexes would afford DNA-binding long-lived luminescent probes, as previously reported for lanthanide-binding peptides grafted with proflavine or naphthalimide.[10,11] Non-substituted thiazo compounds were chosen (Fig. 1, R =H) for their large affinity for DNA and their similar fluorescence properties to naphthalimide, which is known to efficiently sensitize the lanthanide europium ion.[11] An acidic group was introduced by the reaction with glycine for efficient coupling to the *N*-terminus of peptides. The two angular and linear derivatives (**6a** and **6b** respectively; Fig. 2) were targeted in order to compare their behaviors with DNA. However, we report herein that cyclisation occurs exclusively in positions 3 and 4 of the naphthalimide moiety affording the angular isomer **6a** only, contrary to what was described in previous reports that did not demonstrate unambiguous proof of the regioselectivity.[7] DNA binding studies indicate that the aromatic moiety found in the angular regioisomer **6a** has promising DNA binding properties with a large fluorescence quenching upon binding and an affinity of 10^4 M^{-1}.

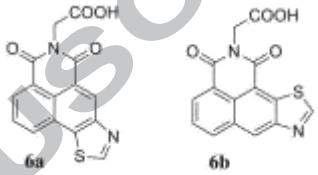
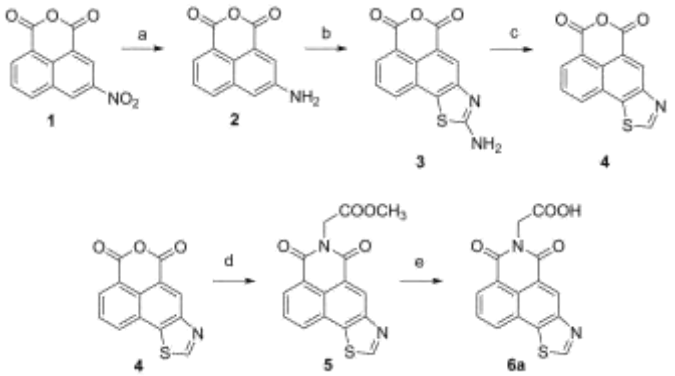


Figure 2. Angular and linear derivatives targeted

The angular thiazonaphthalimide derivative **6a** was obtained in five steps with an overall yield of 8%, starting from the commercially available compound 3-nitro-1,8-naphthalic anhydride **1** (Scheme 1). Detailed experimental conditions for each step and compound characterization are given in the ESI.

The key step of this synthesis is the formation of the aminothiazole ring starting from amino derivative **2**. Compound **2** was obtained by reduction of the nitro group to the corresponding amine, using tin chloride salts in acetic acid at reflux, as described in the literature.[8,12] The formation of the aminothiazole moiety was then carried out by the treatment of **2** with potassium thiocyanate and bromine in acetic acid.[8,13] This synthesis involved a two-step reaction with the initial formation of an aryl-thiocyanate. Indeed, it has been demonstrated that aromatic hydrocarbons bearing strong electron-donating groups such as -NH_2 can react with thiocyanogen[13] to afford aryl-thiocyanates in *ortho* or in *para* positions. Thiocyanogen is formed *in situ* from KSCN and Br_2 according to the method developed by Soderback.[15] The thiocyanate moiety can then react with the amine, leading to the formation of an aminothiazole ring. Regardless of the number of equivalents of bromine and potassium thiocyanate, the order or even the duration of addition for each reagent, only one regioisomer was obtained and isolated, with yields ranging from 60 to 95%. The experimental conditions leading to the highest efficiency are described in the ESI and gave compound **3** in 95% yield. Compound **3** displays limited stability and was therefore quickly engaged in the following step, which consists of deamination involving a diazonium ion. The treatment of **3** with phosphoric acid, sodium nitrite and hypophosphorous acid affords the crude product **4** in 36% yield.[16] Compound **4** was then engaged in a condensation reaction with glycine methyl ester hydrochloride, leading to stable compound **5** in 44% yield after purification on a silica gel column.[17] Finally, saponification with lithium hydroxide provides the desired compound **6a** in 52% yield. The full characterization of stable compound **6a** by ^{1}H and ^{13}C NMR, ESI-MS and microanalysis is reported in the ESI.

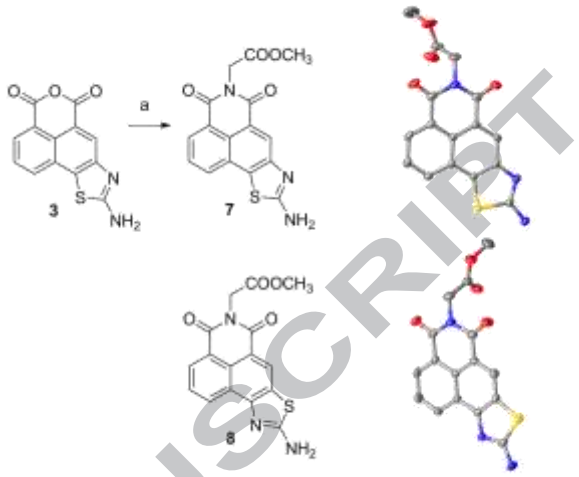


Scheme 1. Reagents and conditions: a) $SnCl_2.2H_2O$ (4 equiv.), HCl (0.74 equiv.), AcOH, reflux, 22 h, quant.; b) KSCN (4 equiv.), Br_2 (1.02 equiv.), AcOH, r.t., 48 h, 95%; c) i) $NaNO_2$ (6 equiv.), H_3PO_4, -10 °C, 1 h 50 min, ii) H_3PO_2 (23 equiv.), r. t., overnight, 36%; d) $H_2NCH_2COOCH_3$•HCl (1.1 equiv.), Et_3N (1.1 equiv.), EtOH, reflux, 5 h, 44%; e) LiOH•H_2O (1.99 equiv.), THF/MeOH, 50 °C, 52%. Overall yield 8%

As the angular structure of compound **6a** cannot be differentiated from the linear structure **6b** by ^{1}H NMR spectroscopy, a NOESY NMR experiment was performed to unambiguously characterize the regioselectivity of the final compound. In the linear compound **6b**, a signature correlation between the proton of the thiazole-fused ring (H_4) and the H_5 proton is expected. By comparison, there should be no correlation between the proton of the thiazole-fused ring (H_2) and the H_5 proton in angular compound **6a**. Therefore the observed correlation pattern (no correlation between H_2 and H_5; see ESI, Fig. S7) evidences the formation of angular compound **6a** with a 3,4-thiazolonaphthalimide moiety.

To unequivocally confirm the regioselectivity of the cyclisation step, the unstable compound **3** was trapped with glycine methyl ester hydrochloride, to afford stable compound **7** in 40% yield. The minor by-product **8** was also isolated in 3% yield (Scheme 2). The two compounds **7** and **8** were fully characterized by ^{1}H and ^{13}C NMR and NOESY experiments. NOE patterns similar to those observed for **6a** confirm the formation of 3,4-thiazonaphthalimide angular regioisomers.

The final proof for this regioselectivity was unambiguously obtained by X-ray crystallography. Indeed, X-ray quality crystals of **7** and **8** were obtained by the slow evaporation of dichloromethane and their crystallographic structures solved by X-Ray diffraction (see ESI, Tables S2 and S3 for details). The solid-state structures confirm the formation of two angular compounds. Compound **7** is the expected angular naphthalimide derivative functionalized by an aminothiazole group with nitrogen in position 3 and sulfur in position 4, whereas the minor compound **8** is a similar derivative with the positions of nitrogen and sulfur atoms inverted, i.e. sulfur in position 3 and nitrogen in position 4 (Scheme 2). The formation of this by-product can be explained by the presence of ~10% 4-nitro-1,8-naphthalic anhydride in the commercially available 3-nitro-1,8-naphthalic anhydride, as evidenced by the ^{1}H NMR spectrum of this starting material. The two X-ray structures show intermolecular hydrogen bond networks that result in one dimensional arrangements along the a axis for **7** and the a+b axis for **8**: the protons of the amino groups are H-bonded to one carbonyl ester and one nitrogen atom of the thiazole ring for **7** or to one carbonyl ester and one oxygen atom of the naphthalimide for **8**. The aromatic rings are planar with mean deviations of only 0.024 Å for **7** and 0.018 Å for **8**. The shortest distance between the two thiazonaphthalimide rings were found at 3.412 Å for **7** and 3.438 Å for **8**, between two different molecules related by the symmetry center of the space group ($P2_1/n$ for **7** and P-1 for **8**). These distances are short enough to evidence π-stacking interactions in both structures.



Scheme 2. Reagents and conditions: $H_2NCH_2COOCH_3$.HCl (1.1 equiv.), Et_3N (1.1 equiv.), EtOH, reflux, 5 h, 40%. Crystallographic structures of compounds **7** and **8**.

The literature reports of only 3- and 4-substitution on naphthalic anhydride or naphthalimide may suggest the poor reactivity of position 2 in the aromatic ring due to steric hindrance or electronic effects. This would prevent the reaction of the thiocyanate group at this position and the formation of 2,3-thiazole fused linear compounds. In fact, the reported linear compounds[8] have been characterized by 1D NMR only and therefore lack reliable structural assignment. Notably, the ^{1}H NMR signals of the aromatic moieties in compounds **7** and **8** are very similar to those previously reported (see ESI, Table S1). Therefore, our results based on unambiguous structural proof strongly suggest that the thiazonaphthalimide major compounds previously described by Qian and co-workers[8] are in fact the angular ones, and that their minor product is another angular isomer with reversed positions of the nitrogen and sulfur atoms, formed from an impurity present in the starting material.

Thiazonaphthalimide derivatives **5**, **7** and **8** were studied for their photophysical properties and their interactions with ct-DNA. These ester derivatives are preferred over the acids, in order to avoid electrostatic repulsions between the carboxylate group and the negatively charged phosphates of DNA at physiological pH. The comparison of these three derivatives gives valuable information regarding the influence of the nitrogen and sulfur atoms positions in the ring and the substitution of the thiazole group by a hydrogen or an amine group. The fluorescence of each compound was measured after the addition of increasing amounts of ct-DNA (expressed in base pairs (bp)) and excitation at its maximum absorption wavelength at 298 K in a buffered solution. These results show a decrease of the fluorescence of each aromatic compound after the addition of ct-DNA in solution, reflecting an interaction between the aromatic rings and the double-helix of DNA, as shown in Figure 3A for compound **5** and ESI Fig. S10 and S11 for compound **7** and **8**, respectively.

Two parameters were extracted from these experiments to compare the interactions of the three compounds with ct-DNA. The association constant K_a for the binding of the naphthalimide derivatives to ct-DNA was calculated by fitting the evolution of the fluorescence spectra with a 1:1 binding model using the program Specfit (Fig. 3B). The corresponding K_a values are reported in Table 1 and exemplifies differences in the binding of the three compounds with a significantly larger affinity for the

non-substituted thiazonaphthalimide **5** (K_a = $10^{4.0}$ M^{-1}).[18] The substitution of the thiazole ring by the mesomeric electron-donating amino group decreases the affinity for the double helix by a factor of 2-3.5. Moreover, compound **5** also displays the largest fluorescence quenching upon ct-DNA binding.

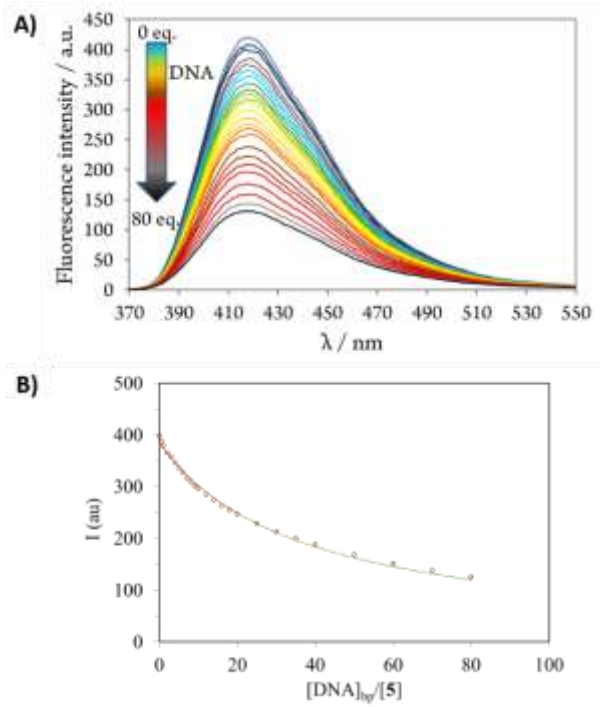


Figure 3. A) Titration of compound **5** (2.5 μM) (HEPES buffer 10 mM, pH 7.0, NaCl 10 mM) at 298 K. Fluorescence upon excitation at 356 nm as a function of ct-DNA (bp). **B)** Corresponding evolution of the fluorescence at 418 nm; circles are experimental points and the line represents calculated data with the program SPECFIT ($logK_a$ = 4.0).

Table 1. Association constants of the naphthalimide derivatives with ct-DNA at pH 7.

Compound	**5**	**7**	**8**
$logK_a$	4.00(3)	3.45(7)	3.67(3)

The melting temperature of double-stranded DNA can be used to partially characterize the mode of interaction of aromatic compounds with DNA.[19] No significant alteration of the melting temperature was observed in the presence of thiazonaphthalimide compounds **5**, **7** and **8**, presumably indicating that none of the three aromatic compounds interacts with DNA *via* an intercalative process between the base pairs of DNA (see ESI, Fig. S12). Indeed, although an intercalative process is mentioned in the literature for the interaction between thiazonaphthalimide derivatives and ct-DNA, little evidence has been provided.

In summary, we have reported the synthesis and unambiguous characterization of angular thiazonaphthalimide derivatives. The formation of linear regioisomers was revealed to not be possible, which is in accordance with the poor reactivity of position 2 in the ring. Compounds **5**, **7** and **8** strongly bind to ct-DNA, with no significant evolution of the ct-DNA melting temperature upon binding, which suggests a non-intercalative process. The largest affinity and fluorescence quenching is observed for the non-substituted ring. The next step will be to graft compound **6a** to amino groups found in peptides or proteins to obtain fluorescent probes sensitive to DNA.

Acknowledgments

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Highlights

- ✓ **Thiazonaphtalimide derivatives were synthesized**
- ✓ **Exclusive formation of linear isomer was demonstrated by NMR and X-ray diffraction.**
- ✓ **Photophysical properties and interaction with ct-DNA were studied**