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DOI: 10.1002/cmdc.200900409

Synthesis and Biological Evaluation of 9-Oxo-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile Analogues as Potential Inhibitors of Deubiquitinating Enzymes

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High-throughput screening highlighted 9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (**1**) as an active inhibitor of ubiquitin-specific proteases (USPs), a family of hydrolytic enzymes involved in the removal of ubiquitin from protein substrates. The chemical behavior of compound **1** was examined. Moreover, the synthesis and in vitro evaluation of new compounds, analogues of **1**, led to the identification of potent and selective inhibitors of the deubiquitinating enzyme USP8.

Introduction

Cysteine proteases, a subfamily of a wider class of protein hydrolytic enzymes, are involved in a series of diseases and pathological conditions such as cancer, neurodegenerative diseases, inflammation, viral infection, and cardiovascular disease. Deubiquitinating enzymes (DUBs), which mainly belong to the cysteine protease family, mediate the removal and processing of ubiquitin, a highly conserved protein expressed in eukaryotic cells, the primary function of which is to mark proteins for proteasomal degradation. DUBs can be divided into five subclasses on the basis of their ubiquitin protease domains; among these, the ubiquitin-specific protease (USP) class is the most represented. The catalytic domain of the USP class contains two short and well-conserved motifs, called Cys and His boxes, which include the residues crucial for catalytic hydrolysis.[1] Owing to their protease activity and their involvement in several human pathologies, USPs are emerging as potential biological targets for pharmacological interference in the ubiquitin regulatory machinery, as demonstrated by the number of patents claiming inhibitors of this system.[2]

Our interest is focused on two specific USP enzymes, namely USP7 and USP8. USP7 (or HAUSP) was shown to associate with the protein Mdm2, an E3 ubiquitin ligase that recognizes the N-terminal transactivation domain of the p53 tumor suppressor and which induces its degradation by ubiquitination. USP7 also interacts directly with p53,[3] FOXO4,[4] PTEN,[5] and several viral proteins,[6] thus connecting this enzyme with essential viral proteins and oncogenic pathways. In addition, phenotypes associated with USP7 silencing strongly suggest that targeting USP7 by small-molecule inhibitors may have the potential for antiviral and anticancer therapies.[7] USP8 (or UBPY) interacts with many substrates, such as the epidermal growth factor receptor (EGFR, essential for the regulation of cell survival, proliferation, and differentiation pathways), and is a key regulator of receptor endocytosis and trafficking.[8]

The aim of our work was the identification of a novel class of potent inhibitors of USP7 and USP8 deubiquitinating enzymes. High-throughput screening of 65 092 chemically diverse compounds for activity toward full-length USP7 cysteine protease in a fluorescence-based biochemical assay identified functionalized cyanopyrazines as active compounds.[9] In particular, 9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (**1**) inhibited deubiquitinating activity with an IC_{50} value in the micromolar range. This compound (prepared in high yield and on the multi-gram scale by reaction between ninhydrin and diaminomaleonitrile)[10] has already been reported to be a charge-transporting material for electrophotographic photoreceptors,[11] a useful tool for the fluorescence detection of α-amino acids,[12] and a potential anti-neoplastic agent.[13] Our goal is the synthesis of new analogues of compound **1**, with the aim of increasing potency and selectivity.

Results and Discussion

Chemistry

Starting with the chemical structure of **1**, three different variation points were identified: introduction of substituents on the phenyl ring, transformation of cyano group(s), and functionalization of the ketone moiety (Figure 1).

The introduction of substituents on the phenyl ring of **1** was achieved by a three-step protocol, starting from 1-indanone, as shown in Scheme 1. The first step was the oxidation of 1-indanones to 1,2-indandiones. This was carried out by the formation of an oxime derivative at the α-position of the ketone

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.200900409.

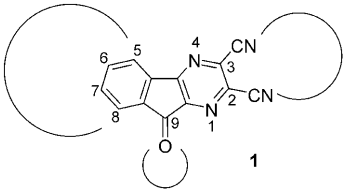
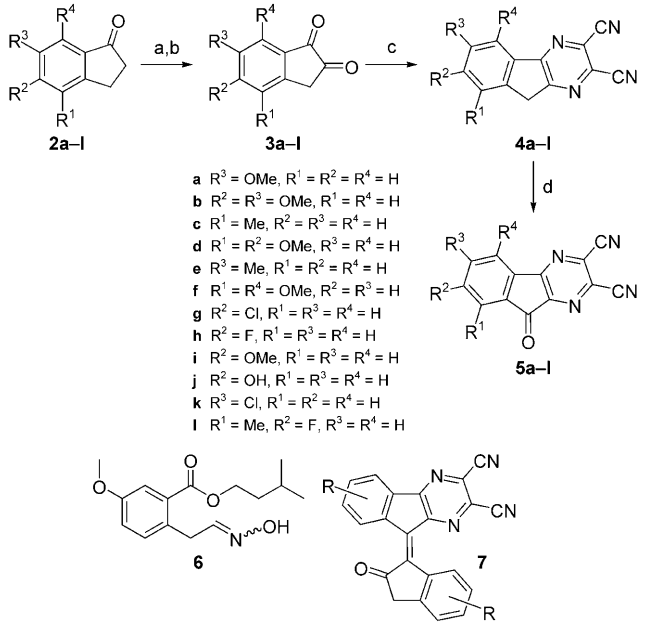


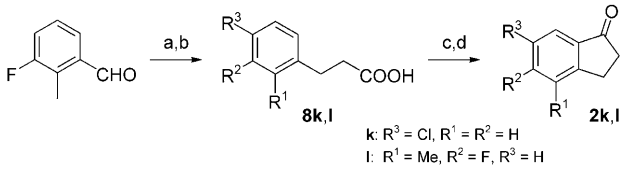
Figure 1. Structure and functionalization approaches of compound **1**.



Scheme 1. Synthesis of compound **5** and structures of by-products **6** and **7**. Reagents and conditions: a) isopentyl nitrite, HCl (concd), MeOH, 40 °C; b) CH_2O (aq), HCl (concd); c) DAMN, *i*PrOH; d) $K_2Cr_2O_7$, AcOH, H_2O, 100 °C.

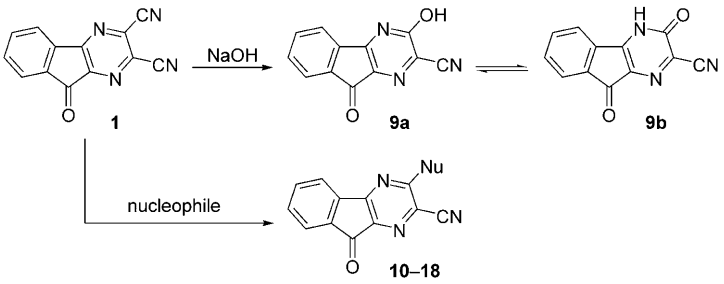
moiety and its subsequent hydrolysis. Unfortunately, the protocol for preparation of the oxime intermediates, involving deprotonation with strong bases such as NaH and reaction with isopentyl nitrite, afforded the expected products in very low yields. The main undesired by-product, as reported for the reaction of 6-methoxy-1-indanone **2a**, is the open derivative **6**,[14] in accordance with the mechanism proposed by Jha and coworkers.[15] Better yields for the formation of the desired oximes were obtained by using a mixture of isopentyl nitrite and concentrated hydrochloric acid in methanol under gentle warming.[16] Subsequent acidic hydrolysis, in the presence of formaldehyde,[17] led to the 1,2-indandione derivatives **3** in high yields, which were cyclized with diaminomaleonitrile (DAMN) in isopropanol at room temperature. Although complete conversions were observed in the cyclization step, the isolated yields were modest due to the formation of a self-condensation by-product **7**,[18] as previously reported by Popp.[10] Finally, intermediates **4** were selectively oxidized at position 9 (methylenic group) with potassium dichromate in hot acetic acid. Following this synthetic protocol, 12 new derivatives **5a–l**, analogues of compound **1** (Scheme 1), were prepared and tested.

1-Indanones **2a–j** are commercially available, whereas compounds **2k** and **2l** were obtained by intramolecular Friedel–Crafts acylation from the corresponding phenylpropionic acids **8k,l**. Compound **8l** was prepared easily by starting from the commercially available 3-fluoro-2-methylbenzaldehyde, through a Knoevenagel condensation with malonic acid, and subsequent hydrogenation of the C=C double bond (Scheme 2).



Scheme 2. Synthesis of indanones **2k,l**. Reagents and conditions: a) malonic acid, piperidine, pyridine, reflux, 83%; b) H_2, Pd/C, MeOH, quant.; c) $(COCl)_2$, CH_2Cl_2; d) $AlCl_3$, CH_2Cl_2, 40 °C.

As far as the transformation of the pyrazine portion of the molecule is concerned, the two cyano groups showed peculiar reactivity. For example, any attempt at hydrolysis failed. Treatment of **1** with aqueous hydrochloric acid, even after prolonged periods at reflux, afforded only unreacted starting material. On the other hand, compound **1** quickly reacted in basic aqueous solution to give derivative **9** by nucleophilic replacement (Scheme 3). Between the two possible tautomers **9a** and



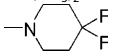
Scheme 3. Nucleophilic replacement of a cyano group of **1**; see Table 1 for more details.

9b, the second one was favored, as shown by both single-point calculations and NMR spectroscopy.

The chemical lability of one nitrile group has been previously reported to result from nucleophilic displacement of symmetric 2,3-dicyanopyrazines by lower alcohols in polar aprotic media.[19] This reactivity could be due to the deactivation given by the two heteroatoms causing the halide-like behavior of the cyano group. Other nucleophiles (O, N, C, and S nucleophiles) gave the same replacement, thus allowing the synthesis of new analogues of **1**, reported with the corresponding reaction conditions in Scheme 3 and Table 1.

Because of the asymmetry of **1**, two possible products may, in principle, be obtained by nucleophilic replacement of cyano groups. Despite this, all the synthesized products were recov-

Table 1. Nucleophiles and conditions used for the derivatization of compound **1**.

Nucleophile	Conditions	Product
–OMe	MeOH, Na, RT	**10**
–OEt	EtOH, Na, RT	**11**
$-O(CH_2)_2OCH_3$	Methoxyethanol, MW, 150 °C	**12**
$-NH_2$	NH_4OAc, THF, 70 °C	**13**
$-N(CH_3)_2$	Dimethylamine, THF, RT	**14**
	4,4-Difluoropiperidine, THF, RT	**15**
–NHCN	Cyanamide, NaH, DMF, RT	**16**
	Ethylcyanoacetate, NaH, DMF, RT	**17**
–SEt	EtSH, Na, THF, RT	**18**

ered as single compounds, evidence of a different reactivity between the two cyano moieties.

X-ray diffraction of compound **11** (Figure 2) confirmed that the substitution occurred at position 3, as indicated in Scheme 3.[20] The increased lability and the sensitivity to nucleophilic displacement of the cyano group are probably due to the electronic delocalization given by the carbonyl moiety at the *para* position, which could stabilize a negative charge. This hypothesized electronic stabilization is supported by ab initio full-geometry optimization of compound **1** performed by MNDO,[21] Hartree–Fock,[22] and DFT[23] (B3LYP[24] functional) calculations at the 6-31G(d)[25] level using GAMESS.[26] Estimation of partial atomic charges of compound **1** was performed considering Mulliken[27] and Löwdin[28] population analysis (enumeration of atoms for the estimation is depicted in Figure 3). Although Mulliken and Löwdin methods do not converge to a constant value, calculated partial atomic charges (Table 2) confirm high electrophilicity at C12, which drives the regioselective nucleophilic displacement at C12 instead of C11.

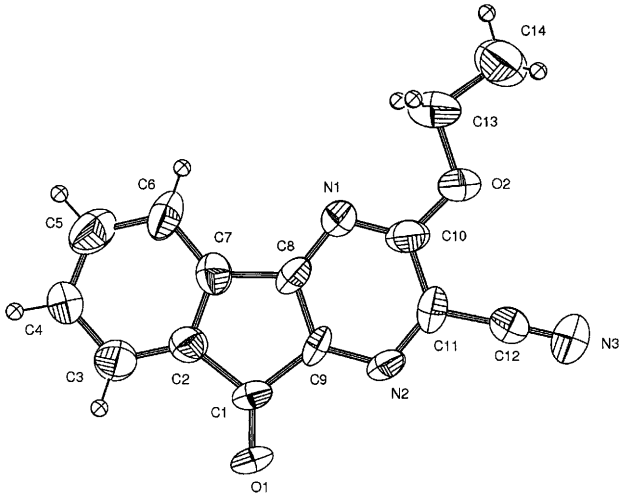


Figure 2. ORTEP plot of the molecular structure of compound **11**.

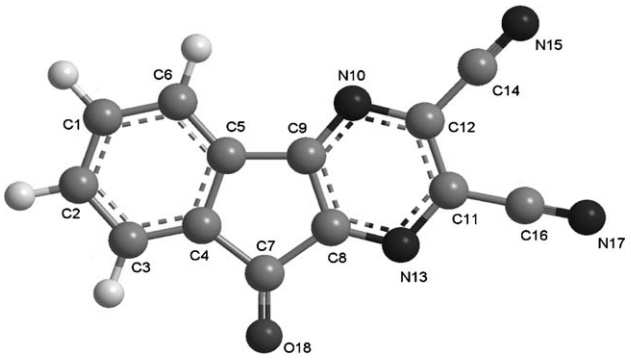


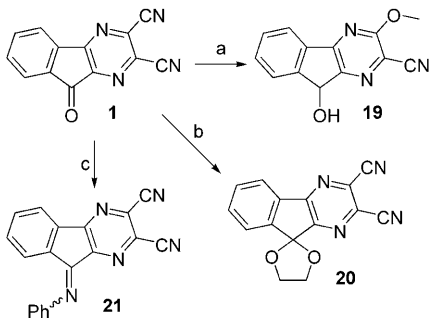
Figure 3. Enumeration of atoms of compound **1** for ab initio partial charges estimation.

Table 2. Mulliken and Löwdin atomic charges estimation of C11 and C12 atoms.

		Mulliken charges		Löwdin charges	
Atom	MNDO	RHF/ 6-31G*	DFT B3LYP/ 6-31G*	RHF/ 6-31G*	DFT B3LYP/ 6-31G*
C11	0.087	0.260	0.308	0.045	0.039
C12	0.127	0.295	0.334	0.075	0.054

The third point of functionalization of **1** is the carbonyl moiety present at the junction of the two aromatic rings. As a first possible modification, reduction of the ketone to a hydroxy group was considered. Unfortunately, due to the peculiar reactivity of the cyano group at position 3, classical hydride-mediated reduction of the carbonyl group in protic solvent was not possible. In fact, the mixture of sodium borohydride and methanol promoted the replacement of the cyano group at position 3 of compound **1** with a methoxy group. Only after this replacement, was the carbonyl moiety reduced, giving the final product **19** that features a hydroxy group at position 9 (Scheme 4).

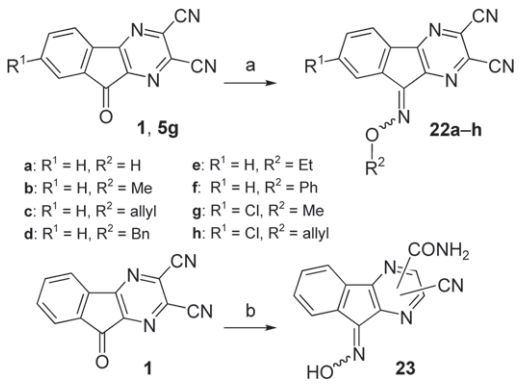
Various functionalizations of the ketone moiety were then considered, that is, ketals, oximes, and imines. Ketal **20**, obtained by reaction with ethylene glycol under acidic conditions, and imine **21**, prepared with aniline under microwave ir-



Scheme 4. Synthesis of compounds **19**–**21**. Reagents and conditions: a) $NaBH_4$, MeOH, 0 °C, 53%; b) ethylene glycol, PTSA, toluene, reflux, 64%; c) aniline, toluene, MS, MW, 150 °C, 60%.

radiation, were synthesized by starting from compound **1** (Scheme 4), whereas the oxime derivatives were prepared both from **1** and from **5g**.

Oxime and *O*-alkyloxime derivatives **22a–h** were synthesized by direct reaction of **1** and **5g**, respectively, with hydroxyamine hydrochloride and *O*-alkylhydroxyamine hydrochloride in pyridine as solvent (Scheme 5). As expected, all the prepared



Scheme 5. Synthesis of compound **22** and proposed structure for **23**. Reagents and conditions: a) NH_2OH·HCl or *O*-alkylhydroxyamine·HCl, pyridine, MS; b) NH_2OH (50% in H_2O), CH_3CN, 0 °C, 62%.

oxime compounds were obtained as mixtures of *E* and *Z* isomers at a variable ratio.

A peculiar behavior was observed in the treatment of compound **1** with 50% hydroxyamine in water and acetonitrile as solvent. LC–MS analysis of the obtained product showed not only the formation of the oxime moiety, but also partial hydrolysis of one of the cyano groups to primary amide (Scheme 5, compound **23**). NMR analysis confirmed the presence of a single compound, but it could not confirm the regiochemistry, which is still unassigned.

In vitro screening

Human USP7 and USP8 enzymes were overexpressed in their full-length forms as functional enzymes in baculovirus-infected insect cells and purified using the His-tag affinity chromatography procedure as previously described.[9] Inhibition of USP7 and USP8 deubiquitinating activity was evaluated by using ubiquitin C-terminal 7-amido-4-methylcoumarin (Ub–AMC), which is hydrolyzed by deubiquitinating enzymes, thus releasing AMC; this can be monitored by fluorescence spectroscopy.[29]

IC_{50} values (Table 3) were calculated from a dose–response curve after dilution of the synthesized derivatives in eight final concentrations, ranging from 100 μM to 10 nM. As reported in Table 3, the products that arise from replacement of the cyano group at position 3 of the pyrazine ring are not active (compounds **10**–**18**), except for compound **9**, which bears a carbonyl group at position 3; this compound is active toward USP7 but is sixfold less potent than the reference compound **1**. These results support the fundamental importance of the cyano group at position 3 as a strong hydrogen bond acceptor.

Table 3. Inhibitory effect of synthesized compounds on USP7 and USP8 deubiquitinating activity.

Compd	IC_{50} [μM] USP7	USP8	Compd	IC_{50} [μM] USP7	USP8
1	3.5	0.29	**14**	>100	>100
5a	>100	4.0	**15**	>100	>100
5b	10.2	2.5	**16**	>100	>100
5c	>100	3.1	**17**	>100	>100
5d	18	0.71	**18**	>100	46
5e	7.2	0.93	**19**	AF[a]	AF[a]
5f	12.7	0.81	**20**	>100	13
5g	0.4	0.096	**21**	4.1	0.35
5h	4.3	0.25	**22a**	>100	7.0
5i	>100	2.1	**22b**	>100	0.98
5j	66	>100	**22c**	>100	0.56
5k	2.55	0.133	**22d**	>100	0.85
5l	2.22	0.316	**22e**	>100	0.28
9	21.8	8.4	**22f**	>100	0.24
10	>100	31	**22g**	0.58	0.103
12	>100	53	**22h**	0.767	0.13
13	>100	48	**23**	13	0.73

[a] Autofluorescent.

Among the analogues functionalized on the phenyl ring, the most interesting compounds are characterized by the presence of a halogen atom (chlorine or fluorine, compounds **5g**,**h**,**k**,**l**). The most active one, **5g**, features a chlorine atom at position 7 and is about ninefold more potent than **1** toward USP7. Previous kinetic data on USP7 allowed the identification of an uncompetitive reversible inhibition mechanism for **5g**. This compound was also shown to inhibit the USP7-mediated removal of ubiquitin from p53 protein, thus confirming that this chemical series is able to inhibit the deubiquitination of physiological substrates.[9]

In contrast, analogues bearing methyl, methoxy, or hydroxy groups are less active than the reference compound. The introduction of moieties that break the aromaticity and the planarity of the whole system, such as compound **20**, in which the dioxolane group lies perpendicular to the plane of the tricyclic scaffold, leads to loss of activity.

The other two derivatizations of the carbonyl moiety (oximes and imines) preserve the planarity of the tricyclic system and the conjugation of the exocyclic heteroatomic double bond, but are characterized by a higher steric demand than the simple carbonyl group, because of the presence of an alkyl/aryl group on the heteroatom. Moreover, in the case of oxime derivatives, an additional heteroatomic center is present, which is able to form a hydrogen bond. Among these, phenylimide **21** shows the same activity of reference compound **1**, and *O*-alkyloxime derivatives of chloro compound **5g** (compounds **22g**,**h**) are 2–6-fold more active than **1**, both toward USP7 and USP8. Interestingly, the removal of the chlorine atom on the phenyl ring of *O*-alkyloxime derivatives of **1** allowed us to generate products that become totally inactive toward USP7, but which remain active at the sub-micromolar level toward USP8 (compounds **22b**–**f**). This selectivity was con-

firmed by assessing the inhibitory effect of these compounds against an extended panel of cysteine proteases. Among these, as illustrated in Figure 4, the representative compound

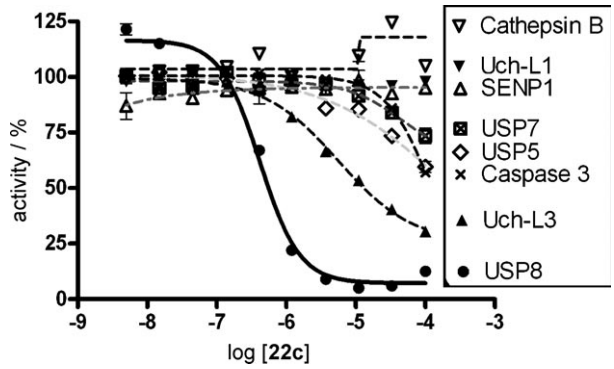


Figure 4. Dose–response curves for compound **22c** toward a representative panel of cysteine proteases (in vitro fluorescence assays).

22c showed sub-micromolar activity only at USP8 and displayed partial activity against Uch-L3. These new derivatives (compounds **22b**–**f**) were next evaluated for their efficacy in cancer cells; they were found to affect the viability of HCT116 colon and PC-3 prostate cancer cell lines with IC_{50} values in the 0.5–1.5 μM range (data not shown). Importantly, the presence of a primary amide group on the pyrazine ring (compound **23**) partially restored activity at USP7, although an H-oxime moiety was present (compound **22a** was inactive on USP7). Cell-based studies of the on-target effects of these USP8-specific compounds are currently in progress.

Conclusions

As result of this preliminary work, the chemical behavior and the in vitro screening of the 9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile compound class were evaluated, to obtain a representative collection of analogues with interesting activity as potential inhibitors of ubiquitin-specific proteases.[30] Among these derivatives, compounds featuring a halogen atom—chlorine and fluorine—on the phenyl ring showed activity in the sub-micromolar range toward USP7 and USP8. Moreover, the introduction of *O*-alkyloxime moieties at position 9 of the tricyclic scaffold resulted in the identification of the first specific compounds for USP8 in comparison with five other ubiquitin/ubiquitin-like proteases, thus yielding evidence that small molecules targeting such enzymes are able to inhibit the USP of interest in a selective manner.

Experimental Section

Reagents obtained from commercial sources were used without further purification. Flash chromatography was performed using Merck silica gel 60, 230–400 mesh. All yields are not optimized. ^{1}H NMR spectra were recorded in $CDCl_3$ or [D_6]DMSO as indicated, using a Bruker Avance II 300 MHz instrument. MS data were recorded with a micromass ZQ Waters spectrometer using positive ESI ionization method. Elemental analyses were recorded with a Carlo Erba EA1108 instrument. Microwave (MW)-assisted reactions were performed in sealed tubes with an Emrys Optimizer Biotage MW oven.

General procedure A: synthesis of compounds 5a–l. Isopentyl nitrite (0.73 mL, 5.5 mmol) and HCl (37%, 0.5 mL) were added to a suspension of substituted 1-indanone (5 mmol) in MeOH (12 mL) warmed to 40 °C. After 1 h at 40 °C, the precipitate was collected by filtration, washed with MeOH, and dried under vacuum. The solid obtained was suspended in CH_2O (36% aqueous, 1.6 mL) and HCl (37%, 3.2 mL), and the mixture was stirred at room temperature for 16 h. H_2O (20 mL) was added, and the suspension was extracted with CH_2Cl_2 (3×15 mL). The collected organic phases were dried over Na_2SO_4, filtered, and evaporated. The crude product was used without further purification. A suspension of DAMN (324 mg, 3 mmol) in *i*PrOH (15 mL) was added to a suspension of substituted 1,2-indanone **3** (3 mmol) in *i*PrOH (15 mL). The mixture was stirred at room temperature for 24 h, and then the precipitate was collected by filtration, washed with EtOH, and dried under vacuum. A suspension of $K_2Cr_2O_7$ (434 mg, 1.44 mmol) in AcOH (0.8 mL) and H_2O (0.2 mL) was added to a suspension of compound **4** (0.8 mmol) in AcOH (1.6 mL). The mixture was slowly heated to 100 °C and was vigorously stirred at this temperature for 1 h. The hot suspension was poured into H_2O (10 mL), and the precipitate was collected by filtration, washed with H_2O, and dried under vacuum.

6,7-Dimethoxy-9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (5b). Prepared according to general procedure A in 32% yield (over four steps) as a red solid. ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta =$ 7.58 (s, 1H), 7.48 (s, 1H), 4.03 (s, 3H), 3.97 ppm (s, 3H); ^{13}C NMR (75 MHz, [D_6]DMSO): $\delta =$ 185.19, 161.52, 157.15, 154.61, 152.23, 134.21, 133.99, 131.07, 130.97, 114.98, 114.64, 107.73, 106.18, 57.46, 57.07 ppm; ESIMS (+) calcd for $C_{15}H_8N_4O_3$: 292.06, found: 293.0 $[M+H]^+$; Anal. calcd (%): C 61.6, H 2.8, N 19.2, found: C 61.3, H 2.4, N 19.0.

7-Chloro-9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (5g). Prepared according to general procedure A in 19% yield (over four steps) as a yellow solid; mp: 220 °C (dec); ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta =$ 8.13 (d, 1H), 8.04 (br s, 1H), 7.97 ppm (br d, 1H); ESIMS (+) calcd for $C_{13}H_3ClN_4O$: 266.00, found: 266.9 $[M+H]^+$; Anal. calcd (%): C 58.6, H 1.1, N 21.0, found: C 58.4, H 1.4, N 21.3.

7-Hydroxy-9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (5j). Prepared according to general procedure A in 22% yield (over four steps) as an orange solid. The product was not purified by precipitation but, after evaporation of the solvent, by flash chromatography (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta =$ 11.66 (br s, 1H), 7.84 (d, 1H), 7.30 (d, 1H), 7.09 ppm (dd, 1H); ^{13}C NMR (75 MHz, [D_6]DMSO): $\delta =$ 184.27, 166.68, 160.31, 152.07, 141.73, 134.32, 132.62, 128.71, 128.30, 121.77, 114.84, 114.65, 110.25 ppm; ESIMS (+) calcd for $C_{13}H_4N_4O_2$: 248.03, found: 249.0 $[M+H]^+$; Anal. calcd (%): C 62.9, H 1.6, N 22.6, found: C 63.1, H 1.4, N 22.3.

6-Chloro-9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (5k). Prepared according to general procedure A in 35% yield (over four steps) as a yellow solid. The product was further purified by flash chromatography (CH_2Cl_2/petroleum ether 7:3). ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta =$ 8.22 (d, 1H), 7.98 (d, 1H), 7.88 ppm (dd, 1H); ^{13}C NMR (75 MHz, [D_6]DMSO): $\delta =$ 185.23, 159.53, 150.86, 142.45, 140.34, 134.90, 134.86, 134.52, 132.98, 127.13, 124.06, 114.70, 114.51 ppm; ESIMS (+) calcd for $C_{13}H_3ClN_4O$: 266.00, found: 267.0

$[M+H]^+$; Anal. calcd (%): C 58.6, H 1.1, N 21.0, found: C 58.2, H 1.0, N 21.0.

2,5-dioxo-2,5-dihydro-1*H*-indeno[1,2-*b*]pyridine-3-carbonitrile (9). A suspension of **1** (5.66 g, 24.3 mmol) in aqueous NaOH (2% *w/v*, 81 mL) was stirred at room temperature for 16 h. The mixture was acidified with 3 N HCl to pH 1, the precipitate was collected by filtration, washed with H_2O, and dried under vacuum to afford **9** (4.88 g, 90%) as a light-brown solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 7.89$ (d, 1H), 7.79–7.62 ppm (m, 3H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 185.78$, 158.15, 157.35, 135.52, 135.37, 134.62, 134.42, 129.30, 125.57, 124.10, 123.84, 116.44 ppm; ESIMS (+) calcd for $C_{12}H_5N_3O_2$: 223.04, found: 224.0 $[M+H]^+$.

3-Methoxy-9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2-carbonitrile (10). Sodium (110 mg) was added to a suspension of **1** (1.10 g, 4.7 mmol) in MeOH (47 mL), and the mixture was stirred at room temperature for 16 h. The precipitate was filtered, washed with EtOH, and dried under vacuum to yield **10** (1.03 g, 93%) as a yellow–green solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 7.92$ (d, 1H), 7.83 (m, 2H), 7.71 (dd, 1H), 4.25 ppm (s, 3H); ESIMS (+) calcd for $C_{13}H_7N_3O_2$: 237.05, found: 238.0 $[M+H]^+$.

3-Ethoxy-9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2-carbonitrile (11). Prepared according to the procedure described for compound **10** in 89% yield as a pale-yellow solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta =$ 7.95 (d, 1H), 7.84 (m, 2H), 7.70 (dd, 1H), 4.53 (q, 2H), 1.34 ppm (t, 3H); ESIMS (+) calcd for $C_{14}H_9N_3O_2$: 251.07, found: 252.0 $[M+H]^+$.

3-Amino-9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2-carbonitrile (13). A mixture of **1** (201 mg, 0.86 mmol), $NH_4CO_2CH_3$ (331 mg, 4.3 mmol), and Na_2SO_4 (200 mg) in THF (2.9 mL) was stirred at 70 °C in a sealed tube for 18 h. The solvent was evaporated, H_2O (5 mL) was added, and the precipitate was filtered, washed with H_2O, and dried under vacuum to afford **13** (171 mg, 90%) as a green solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 8.43$ (br s, 2H), 7.83–7.71 (m, 3H), 7.71–7.59 ppm (m, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 187.31$, 163.58, 158.71, 138.83, 136.51, 136.44, 136.07, 133.66, 124.04, 122.47, 116.41, 110.55 ppm; ESIMS (+) calcd for $C_{12}H_6N_4O$: 222.05, found: 223.1 $[M+H]^+$.

3-(4,4-Difluoropiperidin-1-yl)-9-oxo-9*H*-indeno[1,2-*b*]pyrazine-2-carbonitrile (15). 4,4-Difluoropiperidine·HCl (249 mg, 1.58 mmol) was dissolved in 1 N NaOH (5 mL) and extracted with CH_2Cl_2 (2× 5 mL). The organic phase was dried over Na_2SO_4, filtered, and evaporated. The residue was dissolved in THF (2 mL), and this solution was added to a solution of **1** (185 mg, 0.79 mmol) in THF (2 mL); the mixture was stirred at room temperature for 48 h. The solvent was evaporated, the crude solid washed with EtOH and dried under vacuum to afford **15** (245 mg, 95%) as a yellow–brown solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 7.89$ (d, 1H), 7.79 (dd, 1H), 7.78 (d, 1H), 7.69 (dd, 1H), 4.13 (m, 4H), 2.22 ppm (m, 4H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 187.07$, 161.33, 156.91, 138.57, 137.40, 136.56, 136.30, 133.93, 124.27, 122.76, 122.69, 118.03, 111.93, 44.69, 33.86 ppm; ESIMS (+) calcd for $C_{17}H_{12}F_2N_4O$: 326.10, found: 327.1 $[M+H]^+$.

2-Cyano-9-oxo-9*H*-indeno[1,2-*b*]pyrazin-3-yl-cyanamide (16). Under N_2 atmosphere, CN_2H_2 (44 mg, 1.037 mmol) was dissolved in dry DMF (1 mL), and NaH (21 mg, 0.519 mmol) was added in one portion. After 20 min, a solution of **1** (96 mg, 0.415 mmol) in dry DMF (2 mL) was added dropwise. After 1 h the solvent was evaporated, and the crude was purified by flash chromatography (CH_2Cl_2/MeOH 8:2) to afford **16** (84 mg, 82%) as an orange solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 7.85$ (ddd, 1H), 7.77 (ddd, 1H), 7.76 (m, 1H), 7.67 ppm (ddd, 1H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 186.84$, 165.35, 163.62, 139.32, 136.75, 135.24, 133.50, 132.93, 123.51, 121.99, 119.01, 117.77, 117.57 ppm; ESIMS (+) calcd for $C_{13}H_5N_5O$: 247.05, found: 248.1 $[M+H]^+$.

9-(1′,3′-Dioxolan-2′-yl)-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (20). Ethylene glycol (2.4 mL, 43.8 mmol) and PTSA (6.25 g, 32.8 mmol) were added to a suspension of **1** (5.09 g, 21.9 mmol) in toluene (146 mL). The mixture was held at reflux in a Dean–Stark apparatus for 28 h, and then the solvent was evaporated. The crude was purified by flash chromatography on silica (CH_2Cl_2) to afford **20** (3.87 g, 64%) as a light-yellow solid. ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 8.03$ (m, 1H), 7.83–7.70 (m, 3H), 4.47 ppm (s, 4H); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta = 162.05$, 155.41, 144.27, 135.19, 134.90, 134.40, 133.14, 131.44, 126.10, 123.60, 115.06, 114.80, 106.26, 67.00 ppm; ESIMS (+) calcd for $C_{15}H_8N_4O_2$: 276.06, found: 277.2 $[M+H]^+$; Anal. calcd (%): C 65.2, H 2.9, N 20.3, found: C 65.0, H 2.6, N 20.5.

General procedure B: synthesis of alkyloxyimines 22a–h. NH_2OH·HCl (134 mg, 1.94 mmol) was added to a suspension of **1** (150 mg, 0.646 mmol) in pyridine (10 mL) at 0 °C. Molecular sieves (MS) were added, and the mixture was stirred at room temperature for 16 h or at 60 °C for 1.5 h. The insoluble residue was filtered, the solvent was evaporated, and the crude was purified by flash chromatography.

9-Allyloxyimino-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (22c). Prepared according to general procedure B (column eluent: petroleum ether/EtOAc 9:1) in 15% yield as a yellow solid in diastereomeric ratio 1:1. ^{1}H NMR (300 MHz, [D$_6$]DMSO; mixture of *syn*/*anti* diastereomers): $\delta = 8.43$ (m, 1H), 8.22 (m, 1H), 7.90–7.80 (m, 2H), 6.20 (m, 1H), 5.47 (m, 1H), 5.35 (m, 1H), 5.13 (ddd, 2H), and $\delta = 8.12$ (m, 1H), 7.96 (m, 1H), 7.80–7.69 (m, 2H), 6.14 (m, 1H), 5.53 (m, 1H), 5.38 (m, 1H), 5.08 ppm (ddd, 2H); ^{13}C NMR (75 MHz, [D$_6$]DMSO; mixture of *syn*/*anti* diastereomers): $\delta = 155.54$, 151.70, 146.45, 134.94, 134.55, 133.73, 133.60, 132.82, 132.42, 131.40, 129.27, 123.95, 123.88, 122.23, 119.88, 119.44, 115.14, 115.03, 114.90, 78.62, 78.38 ppm; ESIMS (+) calcd for $C_{16}H_9N_5O$: 287.08, found: 288.2 $[M+H]^+$; Anal. calcd (%): C 66.9, H 3.2, N 24.4, found: C 67.2, H 2.9, N 24.5.

9-Benzyloxyimino-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (22d). Prepared according to general procedure B (column eluent: petroleum ether/EtOAc 9:1) in 32% yield as a yellow solid in diastereomeric ratio 2:1. ^{1}H NMR (300 MHz, [D$_6$]DMSO; mixture of *syn*/*anti* diastereomers): $\delta = 8.42$ (m, 1H), 8.21 (m, 1H), 7.88–7.78 (m, 2H), 7.56–7.49 (m, 2H), 7.47–7.33 (m, 3H), 5.67 (s, 2H), and $\delta = 8.11$ (m, 1H), 7.97 (m, 1H), 7.79–7.69 (m, 2H), 7.56–7.49 (m, 2H), 7.47–7.33 (m, 3H), 5.63 ppm (s, 2H); ^{13}C NMR (75 MHz, [D$_6$]DMSO; mixture of *syn*/*anti* diastereomers): $\delta = 156.22$, 155.57, 151.71, 146.60, 137.15, 134.55, 134.26, 133.25, 132.70, 132.44, 132.15, 131.40, 129.15, 128.98, 128.84, 123.88, 122.25, 115.15, 115.02, 114.89, 79.69, 79.44 ppm; ESIMS (+) calcd for $C_{20}H_{11}N_5O$: 337.10, found: 338.2 $[M+H]^+$; Anal. calcd (%): C 71.2, H 3.3, N 20.8, found: C 71.0, H 3.0, N 23.0.

7-Chloro-9-methoxyimino-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (22g). Prepared according to general procedure B (column eluent: petroleum ether/CH_2Cl_2 1:1) in 65% yield as a light-brown solid in diastereomeric ratio 2:1. ^{1}H NMR (300 MHz, [D$_6$]DMSO; mixture of *syn*/*anti* diastereomers): major product: $\delta = 8.39$ (d, 1H), 8.22 (d, 1H), 7.88 (dd, 1H), 4.42 ppm (s, 3H); minor product: $\delta =$ 8.12 (d, 1H), 7.95 (d, 1H), 7.77 (dd, 1H), 4.37 ppm (s, 3H); ^{13}C NMR (75 MHz, [D$_6$]DMSO; mixture of *syn*/*anti* diastereomers): major product: $\delta = 154.59$, 151.33, 145.32, 145.28, 139.25, 133.43, 133.14, 132.85, 131.26, 128.68, 125.46, 114.95, 114.92, 66.20 ppm; minor

product: $\delta = 155.22$, 151.33, 145.32, 144.67, 138.70, 132.74, 132.32, 131.84, 131.46, 125.53, 122.04, 114.99, 114.79, 66.20 ppm; ESIMS (+) calcd for $C_{14}H_6ClN_5O$: 295.03, found: 296.0 $[M+H]^+$; Anal. calcd (%): C 56.9, H 2.1, N 23.7, found: C 56.7, H 1.9, N 23.6.

9-Allyloxyimino-7-chloro-9*H*-indeno[1,2-*b*]pyrazine-2,3-dicarbonitrile (22h). Prepared according to general procedure B (column eluent: petroleum ether/CH_2Cl_2 1:1) in 56% yield as a light-brown solid in diastereomeric ratio 2:1. ^{1}H NMR (300 MHz, [D$_6$]DMSO; mixture of *syn*/*anti* diastereomers): major product: $\delta = 8.39$ (d, 1H), 8.23 (dd, 1H), 7.90 (dd, 1H), 6.11–6.34 (m, 1H), 5.45–5.54 (m, 1H), 5.37–5.44 (m, 1H), 5.16 ppm (dt, 2H); minor product: $\delta = 8.13$ (dd, 1H), 7.96 (dd, 1H), 7.78 (dd, 1H), 6.08–6.21 (m, 1H), 5.48–5.56 (m, 1H), 5.34–5.40 (m, 1H), 5.10 ppm (dt, 2H); ^{13}C NMR (75 MHz, [D$_6$]DMSO; mixture of *syn*/*anti* diastereomers): major product: $\delta =$ 154.64, 151.41, 145.64, 139.20, 133.57, 133.48, 133.18, 132.92, 132.35, 131.28, 128.64, 125.53, 120.16, 114.94, 114.92, 78.88 ppm; minor product: $\delta = 155.25$, 151.41, 145.38, 138.75, 133.53, 132.87, 131.90, 131.51, 131.28, 128.64, 125.53, 122.04, 119.75, 115.05, 114.81, 78.75 ppm; ESIMS (+) calcd for $C_{16}H_8ClN_5O$: 321.04, found: 322.1 $[M+H]^+$; Anal. calcd (%): C 59.7, H 2.5, N 21.8, found: C 59.4, H 2.8, N 21.6.

Acknowledgements

*We thank François Bellamy, Laurent Daviet, and Rémi Delansorne for many stimulating discussions, Fabrice Moriaud and Roberto Forlani for calculation of and discussions about partial charges estimation on compound **1**, the analytical department of NiKem Research for support in synthesis activities, and all the staff at Hybrigenics for contributing to this study.*

Keywords: drug discovery · heterocycles · inhibitors · proteases · ubiquitin

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Received: September 30, 2009
Revised: February 2, 2010
Published online on February 23, 2010

