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New anti-viral drugs for the treatment of the common cold

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Abstract—Human Rhinovirus (HRV) is the most important aetiologic agent of common cold in adults and children. HRV is a single-stranded, positive sense RNA virus and, despite the high level of conservation among different serotypes, sequence alignment of viral protease 3C with mammalian protease reveals no homology. Thus, protease 3C is an optimal target for the development of anti-HRV agents. In the present work we investigated the design, the synthesis and the development of new potential reversible inhibitors against HRV protease 3C. Docking studies on the crystallized structure of HRV2 protease 3C led us to the design and the synthesis of a series of 3,5 disubstituted benzamides able to act as analogues of the substrate. We also developed 1,3,5 trisubstituted benzamides where aromatic substitutions on the aryl ring led us to investigate the importance of π–π interaction on the stabilization of protease 3C-inhibitor complex. All structures were tested for enzymatic inhibition on HRV14 protease 3C. Results highlighted the inhibitory activity of compounds 13, 14, and 20 (91%, 81%, and 85% at 10 μM, respectively), with the latter exhibiting an ID_{50} (dose that inhibits 50% of the viral cytopathic effect) on HRV-14 = 25 μg/ml.

1. Introduction

Human Rhinovirus (HRV) is the most important aetiologic agent of the common cold in adults and children. At present, no anti-HRV agent exists and the large number of different serotypes (~105)[1] makes unlikely the development of vaccines. HRV clinical manifestations include nasopharyngeal syndrome with signs as sneezing, rhinorrhea and malaise; usually fever is not present and cough is only mild.[2] HRV is a single-stranded, positive sense RNA virus. First step in replication involves its attachment to the membrane of the host cell via ICAM-1 receptors and its penetration by endocytosis. Following the uncoating of the viral RNA from the capsid, the positive strand is translated into a long polyprotein which undergoes proteolysis by virus-encoded protease 2A and 3C.[3] Protease 2A accomplishes the cleavage of Tyr-Gly pairs but is not essential for the viability of the virus. Despite its overall architecture correlating with trypsine-like serine protease, protease 3C (3CP) is a cysteinyl protease with His-Glu-Cys being the catalytic triad. It hydrolyses Gln-Gly, Gln-Ser and Gln-Ala pairs so it is essential for viral replication.[4] Recent DNA sequencing of 3CP of different serotypes revealed a high degree of homology within the 3C coding region including active site residues.[5] However, sequence alignment of viral 3CP with mammalian protease reveals no homology.[6] Thus, 3CP is an optimal target for the development of anti-viral agents.

Elucidation of 3CP crystal structure together with substrate cleavage specificity studies have facilitated the rational design of inhibitors.

Several peptidic and non-peptidic inhibitors acting as analogues of the substrate have been reported. They

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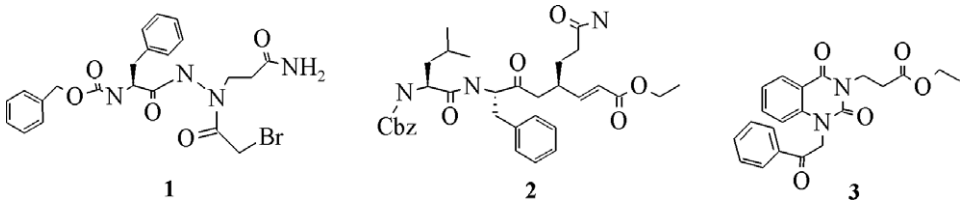


Figure 1. Examples of known HRV 3CP protease inhibitors.

all contain groups which can be attacked by SH of the cysteine of the catalytic triad of the protease, thus generating a reversible analogue of the transition state. It has been found that 3CP needs at least four aminoacids upstream the scissile bond, so tetrapeptides containing electrophiles as aldehydic groups have been synthesised.[7–9] Tetrapeptides, but also tri and dipeptides, reversibly inhibited 3CP with good IC_{50} values. Short polypeptides were also combined with thiol-reactive groups such as methyl and halomethyl ketones[10] (e.g., **1**, IC_{50} = 48 nM, Fig. 1), Michael acceptors[11,12] (e.g., **2**, EC_{50} = 0.54 μM, Fig. 1),[13] iodoacetamides, β-lactones and pseudoxazolones.[14] Peptidic lactam AG7088 was tested in Phase II trial with intranasal delivery. Unfortunately, peptide-based inhibitors show rapid metabolism and poor bioavailability so they are not likely to be good drug candidates.

Non-peptidic 3CP inhibitors include β-lactams,[15] isatines,[16] homophthalimides[17] (e.g., **3**, IC_{50} = 55.4 μM, Fig. 1), alkyl halides, ketones and quinones.[18–20] Various substituted benzamides are also present, in particular, 5-substituted α,β-unsaturated keto benzamides showed good inhibitory properties.[21,22]

The aim of the present study was to identify new non-peptidic low molecular weight broad-spectrum inhibitors of HRV 3CP. Here, it is described the design, the synthesis and the pharmacological evaluation of new potential reversible inhibitors.

2. Drug design

Starting point of molecular modelling studies was the already available X-ray crystal structure of the covalent adduct between HRV-2 3CP and AG7088[23] (pdb code: 1CQQ).

AG7088 is an effective anti-viral agent and inhibits all 48 serotypes of rhinoviruses with Kobs/I = 1,470,000 M^{-1} s^{-1} on HRV-14. It is also equally effective on HRV-serotype 14 (EC_{50} = 0.013μM) and HRV- serotype 2 (EC_{50} = 0.012 μM) in HeLa cells.[23] The catalytic triad of 3CP is represented by Cys 147, Glu 71 and His 40 with the nucleophilic SH of the Cys in the active site covalently linked to the inhibitor's electrophilic β-carbon of the Michael acceptor.[23] Analysis of the HRV-2 3CP-AG7088 X-ray crystal structure suggested that in the catalytic site there are some pockets. A brief exploration highlights that the Michael acceptor is in S3 pocket, the lactamic group leans forward S1 and the benzyl group positions itself in S2 pocket. The lactamic group of the inhibitor is hydrogen bonded both with His 161 and Thr 142 (Fig. 2). Comparing the structure of AG7088 with the structure of 2HN-Thr-Gly-Leu-Phe-Gln-Gly-

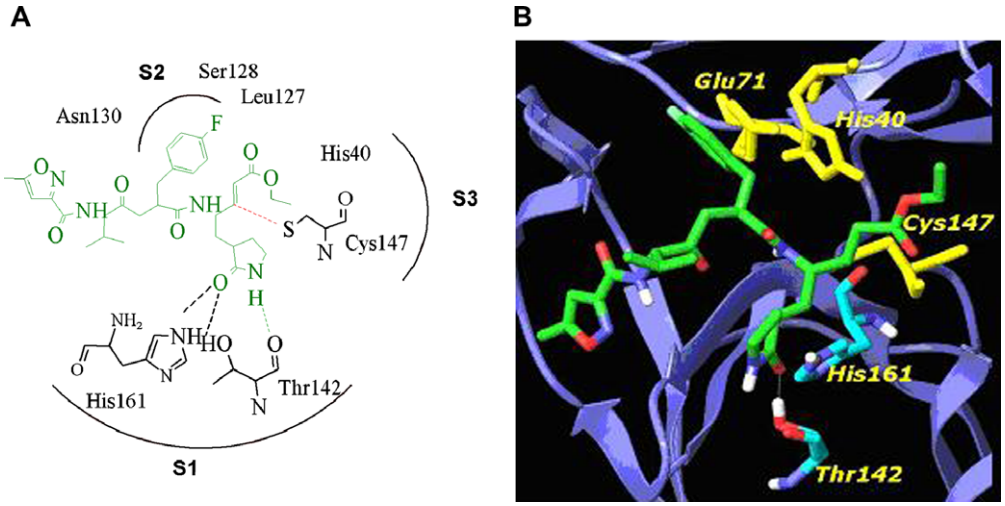


Figure 2. (A) Schematic diagram of AG7088 (green) bound in the HRV-2 3CP active site. Hydrogen bonds are shown as dashed lines (black), covalent bond is shown as red dashed lines and the residues which make up the enzyme binding subsites are depicted. (B) AG7088 bound to HRV-2 3CP. The catalytic triad residues (His40, Glu71, and Cys147 in yellow), the inhibitor AG7088 (green) and hydrogen bonded residues (His161 and Thr142 in turquoise) are shown in tube.

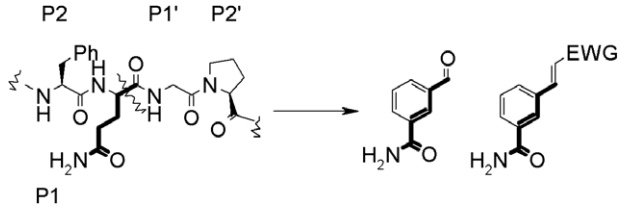


Figure 3. Glutamine-Glycine cleavage site in comparison with 3-formylbenzamide and a Michael acceptor benzamide.

Pro-COOH, a known 3CP substrate that shows high specificity cleavage between Gln (P_1) and Gly (P'_1), the lactamic group obviously mimics the side chain of Gln (P_1).

On the basis of interactions between AG7088 and 3CP other authors designed substituted benzamides.[22] Meta disubstitution allows to mimic the natural substrate Gln (P1) of 3CP as the substituents are at the appropriate distance and orientation (Fig. 3). Amide corresponds to lactamic group in AG7088 and the electrophilic reactive group is always an aldehyde, a ketone or a 1,4-unsaturated ester (Michael acceptor). X-ray crystal structure of a complex between 3-(3-carbamoyl-phenyl)-acrylic acid 2-hydroxyethyl ester and 3CP[21] confirms interactions of carboxamide group with Thr 142 and His 161. Further studies show that inhibitory activity is increased when compounds can bind recognition sites S1–S3 on the 3CP.

We developed a virtual library employing three different core structures: 3-(difluoroacetyl) benzamide (a), 4-(difluoroacetyl)-2,3-dihydro-1H-isoindol-1-one (b) and 4-(difluoroacetyl)-1,3-dihydro-2H-indol-2-one (c) (Fig. 4). The electrophilic reactive group is a difluoromethyl ketone that mimics the Michael acceptor group of AG7088. Positions ortho and meta to difluoromethyl ketone were explored to determine which one is able to place a substituent R, formed by a linker and an aromatic group (Table 1), into the S2 pocket which seems to be essential for good inhibitory activity.

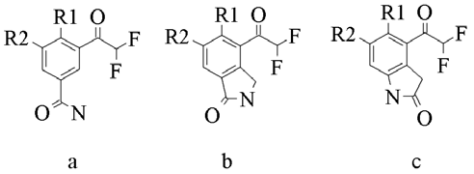


Figure 4. Core structures of the virtual library.

Table 1. Proposed substituents R^1/R^2 and R^3 on the core structures a, b, and c

Compound	R^1/R^2	R^3
Core a, b, and c	$NH–CH_2–R^3$	α-Naphthyl
	$CO–R^3$	Ph
	$CH_2–R^3$	Diphenyl
	$CH_2–CO–R^3$	β-Naphthyl
	$CH_2–CH_2–R^3$	

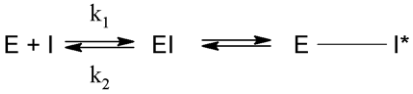


Figure 5. Interaction between 3CP and inhibitor.

The covalent inactivation of 3CP by halomethyl ketones was modelled as shown in Figure 5.

The inhibitor (I) forms first a reversible complex with the enzyme (E) and only then it can undergo a nucleophilic attack by Cys 147, leading to a covalent complex. The observed biological activity depends upon both the equilibrium binding constants (k_2/k_1) and the chemical rate of covalent bond formation. In this study we explored the reversible complex formation as dominated by non-covalent molecular recognition by means of virtual docking and the subsequent chemical reaction as dominated by the reactivity of the halomethyl ketones by means of quantum chemical calculations.

2.1. Virtual docking

All the docking and scoring calculations were performed using the Schrödinger suite of softwares (Schrödinger, LLC, New York, NY). 1CQQ, the co-crystal structure of the covalent adduct between 3CP and AG7088, was used. In order to create the conditions prior to the binding, the covalent bond between the two structures was broken. The side chain of Cys 147, originally bound to AG7088, was shifted to avoid steric clashes and the complex 3CP-AG7088 was energetically minimized with MACROMODEL[24] with the OPLS-AA force field.[25] The minimized complex retains all the original non-bonding interactions.

Docking was performed using GLIDE[26] program (Schrödinger, LLC, New York, NY). Grids for molecular docking were calculated employing different Van der Waals scaling parameters in order to allow the site to 'breathe'. The virtual library of 120 structures previously designed was docked into 3CP structure. For each structure three different poses were sampled and visually inspected to determine which one is able to retain original interactions with Thr 142 and His 161 and to place the aromatic substituent into the S2 lipophilic pocket.

Best poses were retrieved with 3-(difluoroacetyl) benzamide core structure, meta substitution, R group formed by a two-atom linker and phenyl or naphthyl as aromatic group. These results guided chemical synthesis in our laboratory.

2.2. Quantum mechanical calculations

Inactivation power of halomethyl ketones was studied by means of quantum mechanical calculations. We assumed that their biological activity depends upon the chemical rate of covalent bond formation. In fact non-bonded interactions that stabilize EI form and influence the equilibrium binding constants (k_2/k_1) are more or less the same if just the halomethyl ketone structure changes.

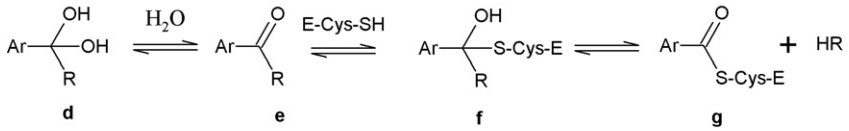


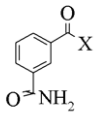
Figure 6. Reactivity of halomethyl ketones.

Halomethyl ketones in water exist in equilibrium with their hydrated form [**d**]. It is clear that only the unhydrated form [**e**] is the active form of the inhibitor that can undergo the nucleophilic attack of the enzyme. As more reactive is the halomethyl ketone and more stable is the hydrated form, the ideal R group should show a compromise: reactive enough to be able to undergo the nucleophilic attack of the Cys, but not too much not to stabilize the inactive hydrated form (Fig. 6).

Ab initio geometry optimization and energy calculations at HF 3-21G* level were performed with Spartan[27] on systems shown in Figure 7 to investigate the chloro and bromo influence on reactivity and subsequently on biological activity of these systems.

Energy and density of LUMO (Lowest Occupied Molecular Orbital) was calculated to evaluate the reactivity of the system towards nucleophilic attack and energy difference between forms [**d**] and [**e**] was employed to evaluate the stabilization of the hydrated form.

CHX_2 and CH_2X pairs with X = Cl or Br were considered. Results are reported in Table 2. Compounds containing chloro show lower LUMO energy and density, that is to say higher reactivity and so hypothetical better biological activity. Moreover, CH_2Br substituent shows a high stability of the hydrated form (about 8 kcal/mol), that means worse biological activity while $CHBr_2$ has a similar stability as $CHCl_2$. According to obtained results, chloro and dichloro methyl ketone should exhibit the best activity, being the right compromise between reactivity and tendency to hydration.



$X = CH_2Cl, CHCl_2, CH_2Br, CHBr_2$

Figure 7. System of ab initio calculations.

Table 2. Hypothesized substituents X and relative energy and density values

SUBST X	LUMO (eV)	ρ LUMO	ΔHF (kcal/mol)
$CHCl_2$	1.51265	0.02815	−22.2
$CHBr_2$	1.58944	0.02774	−21.6
CH_2Cl	1.76881	0.02732	−16.5
CH_2Br	1.81562	0.02722	−24.7

3. Chemistry

The compounds evaluated were isoindolinone derivatives **4** and **5** and benzamide derivatives **6**–**26** (Chart 1 and Table 3).

Compound **4** was prepared in five steps starting from phthalimide **27** which was reduced to isoindolinone **28** with Sn/HCl.[28] Bromination of **28** with Br_2 in the presence of $AlCl_3$ followed by Pd catalyzed coupling with ethyl-1-trimethylstannylvinyl ether and hydrolysis in HCl gave the 6-acetylisoindolinone **30**. Mono bromination of the acetyl group gave the desired compound **4** (Scheme 1).

Compound **5** was prepared from the commercially available 3-bromo-2-methylbenzoic acid **31** which was transformed to the corresponding methyl ester that was brominated at the benzylic position with NBS under

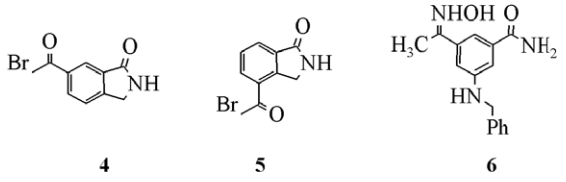
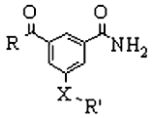
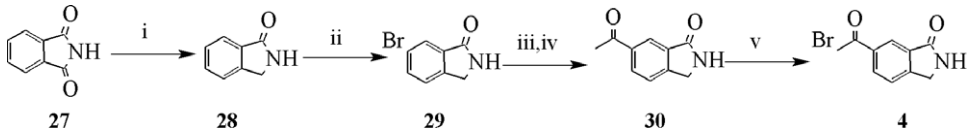


Chart 1.

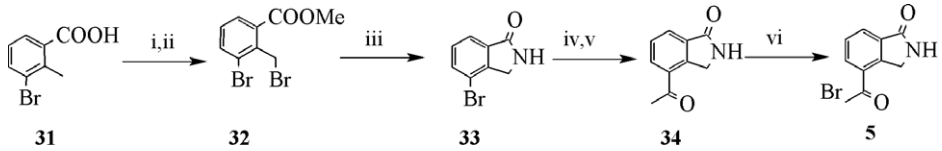
Table 3.



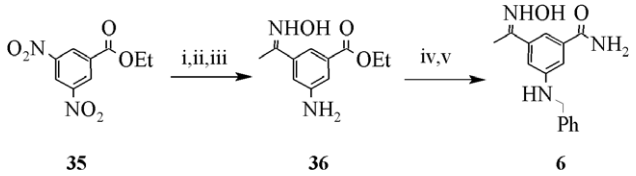
Compound	X	R^1	R
7	H	—	CF_3
8	H	—	CHF_2
9	CH_2	Ph	CHF_2
10	CONH	Ph	CHF_2
11	H	—	$CHCl_2$
12	H	—	CH_2Cl
13	CH_2	Ph	CH_2Cl
14	CH_2	1-Naphthyl	CH_2Cl
15	H	—	$CHBr_2$
16	CH_2	Ph	$CHBr_2$
17	CH_2	1-Naphthyl	$CHBr_2$
18	CONH	Ph	$CHBr_2$
19	CONH	1-Naphthyl	$CHBr_2$
20	H	—	CH_2Br
21	CH_2	Ph	CH_2Br
22	CH_2	1-Naphthyl	CH_2Br
23	CONH	Ph	CH_2Br
24	CONH	1-Naphthyl	CH_2Br
25	NH	Bn	OCH_3
26	NBn	Bn	OCH_3



Scheme 1. Reagents and conditions: (i) Sn, HCl, EtOH, 70 °C; (ii) Br_2, $AlCl_3$, $ClCH_2CH_2Cl$, 70 °C (45% yield); (iii) CH_2=C(OEt)$SnMe_3$, $Pd(PPh_3)_4$, NMP; (iv) HCl (50% yield); (v) Br_2, $CHCl_3$(76% yield).



Scheme 2. Reagents and conditions: (i) MeOH, HCl, Et_2O; (ii) NBS, AIBN, CH_2Cl_2; (iii) NH_3, MeOH (95% yield); (iv) CH_2=C(OEt)$SnMe_3$, $Pd(PPh_3)_4$, NMP; (v) HCl (50% yield); (vi) Br_2, $CHCl_3$ (50% yield).

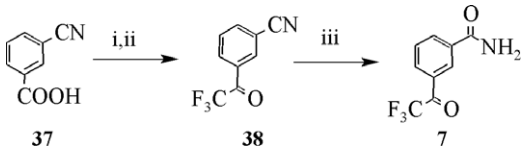


Scheme 3. Reagents and conditions: (i) HCOOH, Et_3N, Pd/C 10%, 20 min.; (ii) $NaNO_2$, 0 °C, 20 min, CH_3CHNOH, $CuSO_4 \cdot 5H_2O$, 10° C, 1.5 h; (iii) $SnCl_2 \cdot 2H_2O$, EtOH, 70 °C (82% yield); (iv) NH_3, 24 h; (v) BnCl, K_2CO_3, reflux (34% yield).

radical condition (AIBN, CH_2Cl_2)[29] to furnish **32**, which in the presence of NH_3 in MeOH gave the 4-bromoisoindolinone **33**.[30] As for compound **4** the Pd catalyzed coupling of **33** with ethyl-1-trimethylstannylvinyl ether followed by hydrolysis with HCl gave the acetyl derivative **34**, which was further brominated to give the desired compound **5** (Scheme 2).

Benzamide derivative **6** was obtained starting from ethyl-3,5-dinitrobenzoate **35** which was selectively reduced with formic acid to the monoamino derivative that was diazotized and reacted with acetaldehyde oxime to furnish the corresponding oxime. Further reduction of the nitro group gave the oxime **36** whose reaction with aqueous ammonia and further benzylation gave **6** (Scheme 3).

Trifluoromethyl derivative **7** was prepared by nucleophilic trifluoromethylation of 3-cyanobenzoic acid **37** followed by oxidation of **38**[31] to benzamide **7** (Scheme 4).

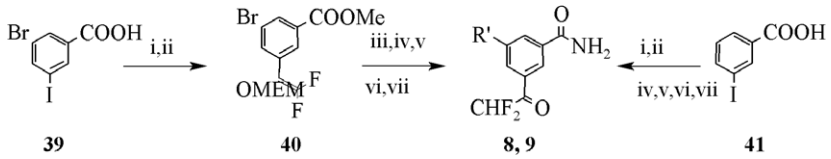


Scheme 4. Reagents and conditions: (i) DMAP, DCC, MeOH (73% yield); (ii) $(CH_3)_3SiCF_3$, CsF, dimethoxyethane (50% yield); (iii) H_2O_2 30%, NaOH 6 N, EtOH, 95 °C (47% yield).

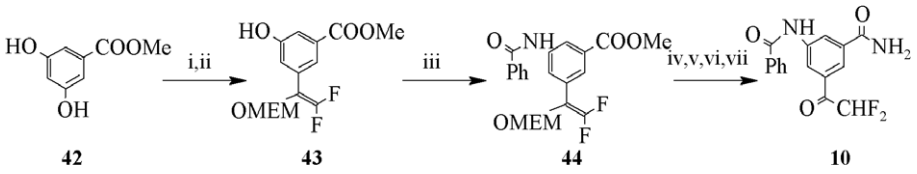
Difluoromethyl derivatives **8**–**10** were prepared starting from 3-bromo-5-iodobenzoic acid **39**, 3-iodobenzoic acid **41** and methyl ester of 3,5-dihydroxybenzoic acid **42** using a palladium catalyzed coupling reaction as a key step for the introduction of the difluoroacetyl group via the corresponding methoxyethoxymethyl (MEM) 2,2-difluoro-1-tributylstannilvinyl ether[32] (Schemes 5 and 6).

The Stille-type reaction with difluorovinylstannane [F_2C=C(OMEM)$SnBu_3$] on the methyl ester of **41** followed by hydrolysis and amidation gave derivative **8**. The methyl ester of **39** was coupled with difluorovinylstannane [F_2C=C(OMEM)$SnBu_3$] to give **40** that with a further palladium catalyzed Negishi-type coupling with a suitable organozinc derivative furnished target compound **9**. The triflate of methyl ester **42** was regioselectively coupled first with difluorovinylstannane [F_2C=C(OMEM)$SnBu_3$] to give **43** which reacted with benzamide under the Buchwald Xantphos catalyzed conditions[33] to furnish **44**. Hydrolysis and amidation gave derivative **10**.

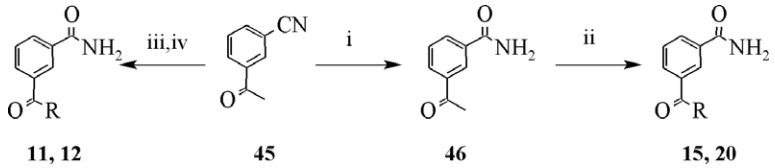
Derivatives **11**, **12**, **15**, and **20** were obtained starting from 3-acetylbenzonitrile **45** (Scheme 7) that was converted into the amide **46**[34] and brominated to give **15** and **20**.[34] Treatment of **45** with TMSCl and MnO_2 in AcOH gave a mixture of mono- and dichloro derivatives that were converted into amides **11**, **12** by hydrolysis with H_2SO_4.



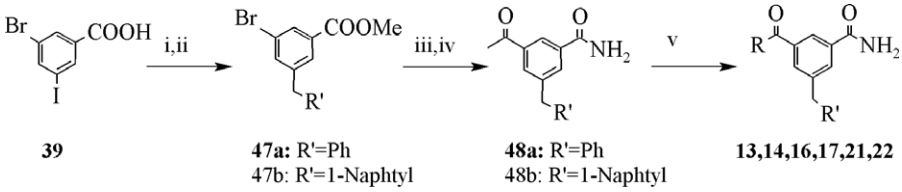
Scheme 5. Reagents and conditions: (i) HCl, Et_2O, MeOH (89% yield); (ii) F_2C=C(OMEM)$SnBu_3$, CuI, PPh_3, $Pd(AcO)_2$, DMF, 60 °C (67% yield); (iii) $Pd(PPh_3)_4$, R^1ZnBr, THF, 70 °C; (iv) Me_3SiCl, MeOH; (v) KOH, EtOH; (vi) $SOCl_2$, 80 °C; (vii) NH_3 (**8** = 70% yield, **9** = 80% yield).



Scheme 6. Reagents and conditions: (i) Tf_2O, Et_3N, CH_2Cl_2 (80% yield); (ii) $F_2C{=}C(OMEM)SnBu_3$ $Pd_2(dba)_3$, LiCl, $AsPh_3$, NMP, 60 °C (50% yield); (iii) $Pd(OAc)_2$, Xantphos, $PhCONH_2$, K_2CO_3, dioxane, 100 °C (33% yield); (iv) Me_3SiCl, MeOH; (v) KOH, EtOH; (vi) $SOCl_2$, 80 °C; (vii) NH_3 (49% yield).



Scheme 7. Reagents and conditions: (i) H_2O_2 30%, NaOEt (67% yield); (ii) Br_2, AcOH (**15** = 15%, **20** = 54%); (iii) MnO_2, $(CH_3)_3SiCl$, AcOH; (iv) H_2SO_4 (**11** = 11%, **12** = 10%).



Scheme 8. Reagents and conditions: (i) HCl 37%, MeOH, 70 °C, 4 h; (ii) R′CH_2ZnBr, $Pd(PPh_3)_4$, THF, 60 °C, 12 h (**47a** = 60%; **47b** = 60%); (iii) BVE, $Pd(OAc)_2$, PPh_3, Et_3N, CH_3CN, 100 °C; HCl 3 N, THF; (iv) NH_3 32%, THF, 80 °C (**48a** = 50%; **48 b** = 50%); (v) Br_2 or Cl_2, $CHCl_3$, 50 °C (**13** = 15%; **14** = 45%; **16** = 65%; **17** = 15%; **21** = 68%; **22** = 52%).

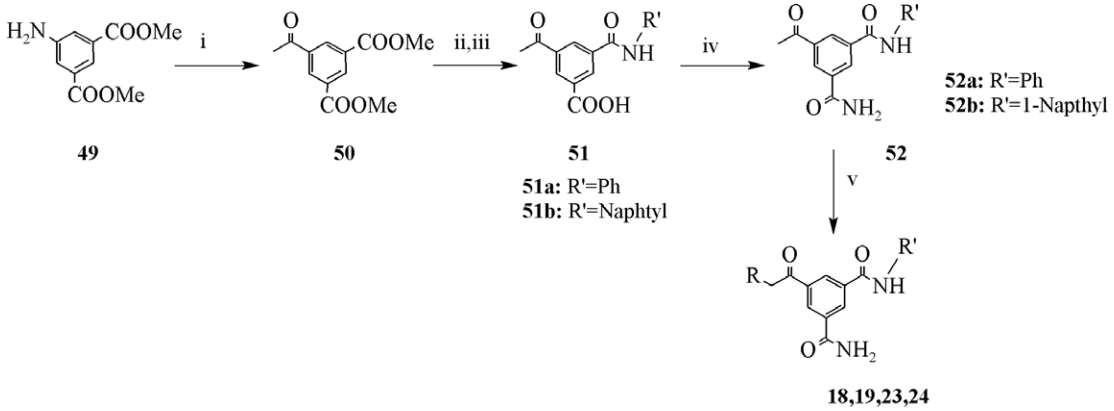
Compounds **13**, **14**, **16**, **17**, **21** and **22** were obtained (Scheme 8) starting from 3-bromo-5-iodobenzoic acid **39**. The Negishi-type palladium catalyzed coupling with organozinc furnished methyl esters **47a**, **b** which were coupled with butylvinyl ether (BVE) in the presence of PPh_3 and $Pd(OAc)_2$ to get after amidation derivatives **48a**, **b**. The last step was the reaction with Br_2 or Cl_2 in $CHCl_3$ to get the desired compounds.

Starting from 5-aminoisophthalate methyl ester **49**, derivative **50**[35] was obtained by a diazotation/acetylation sequence. Basic hydrolysis of **50** followed by mono amidation gave benzoic acid derivatives **51** that were transformed into the corresponding benzamide derivatives **52** which reacted with Br_2 to furnish the target compounds **18**, **19**, **23**, **24** (Scheme 9).

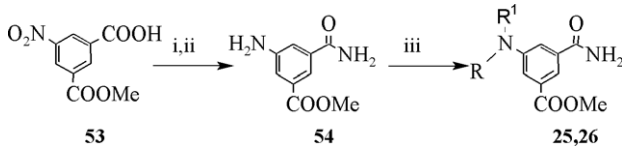
Finally methyl esters **25**, **26** were obtained from nitro benzoic acid **53** by amidation, reduction of the nitro moiety and further benzylation of **54**[36] (Scheme 10).

4. Results and discussion

The aim of the present study was the identification of new potential reversible non-peptidic inhibitors of



Scheme 9. Reagents and conditions: (i) $NaNO_2$, HCl concd, 0 °C, 20′/CH_3CHNOH, $CuSO_4.5H_2O$, 10 °C, 1.5 h/HCl 37%, reflux (42%); (ii) NaOH 2N, reflux; (iii) R′NH_2, DCC, HOBt, DIEA, DMF, 1.30′ (**51a** = 52%; **51b** = 50%); (iv) $SOCl_2$, toluene/THF, NH_3(g) (**52a** = 40%; **52b** = 32%); (v) Br_2, $CHCl_3$ (**18** 65%; **19** 45%; **23** 60%; **24** 68%).



53 54 25,26

Scheme 10. Reagents and conditions: (i) $SOCl_2$, NH_3(g), 3 h; (ii) $SnCl_2 \cdot 2H_2O$, EtOH, 70 °C (50%); (iii) BnCl, Et_3N, reflux, 12 h (**25** = 21%; **26** = 7%).

HRV 3CP. The work started from the consideration that 1,3 disubstituted benzamides behave as good and potent inhibitors of HRV 3CP.[21,22] The amidic group seems to be essential for making hydrogen bonds within the active site of the enzyme, also it resembles the side chain of the natural substrate Gln P_1 at the appropriate distance and orientation. An electrophilic meta group is necessary in order to react with the SH of the cysteine of the catalytic triad of the protease, to generate a reversible analogue of the transition state.

We designed a series of benzamides where the electrophilic group is represented by a methyl ketone alpha mono, di or tri halo-substituted (compounds **7**, **8**, **11**, **12**, **15**, **20**).

Molecular modelling supported the study generating a virtual library where three main structures are represented: 1,3,5 trisubstituted benzamides (compounds **6**, **9**–**10**, **13**, **14**, **16**–**19**, **21**–**26**) and the cyclic derivatives 3,4 and 4,5 isoindolinones (compounds **4** and **5**). Compounds **6**, **9**–**10**, **13**, **14**, **16**–**19**, **21**–**26** all contain an aromatic portion in position 5 that should be able to fill the S2 pocket of the 3CP thus increasing the inhibitory activity. They also contain an amide group in meta to the electrophilic group (halomethyl ketone or oxime). Isoindolinones **4** and **5** are more rigid structures where the electrophile and the amide groups are kept in meta. Quantum mechanical calculations indicated that compounds containing chloro and dichloro methyl ketone would exhibit the best activity, being the right compromise between reactivity and tendency to hydration.

All synthesised compounds were tested on their inhibitory activity against HRV-14 3CP and a colorimetric method[37] for in vitro screening was set up in our laboratories. Prior to testing against 3CP, all compounds were tested for their activity against dithiothreitol (DTT), as many protease inhibitors could be susceptible to free thiol and thus have an unspecific action. None of the tested compounds were inactivated at two different concentrations of DTT (6 and 0.12 mM, data not shown). First set of compounds (**11**, **12**, **15**, **20**) was then screened at 10 μM and each experiment was performed in duplicate. Mean inhibition became $\geqslant$50% using 1 mM leupeptin as control (unspecific protease inhibitor, mean inhibition 57 ± 6%) and this is shown in Figure 8. On the basis of the results, compound **7** was newly synthesised and tested on 3CP in vitro system at various doses, ranging from 0.16 to 100 μM. This compound did not exhibit inhibition of 3CP at any used concentration.

Second set of compounds (**4**–**5**, **6** and **8**–**10**, **13**, **14**, **16**–**19**, **21**–**26**) was screened in duplicate at 10 μM using an Agouron aldehydic compound as reference[38] (named as AL21-01). Data obtained (Fig. 8) show that inhibition of 3CP enzymatic activity is comparable to those of reference compounds **20** (85 ± 6%) and AL21-01 (92 ± 6%) and it was reached for most of the compounds, being very interesting for molecules **4**, **11**, **12**, **13**, **20**, **23**, **24**, **14**, **17**, **22** and **16** (inhibition $\geqslant$70%).

On the basis of these results, 10 molecules were chosen to be further screened at three different concentrations (0.1, 1, 10 μM). Two compounds, **13** and **14**, show an

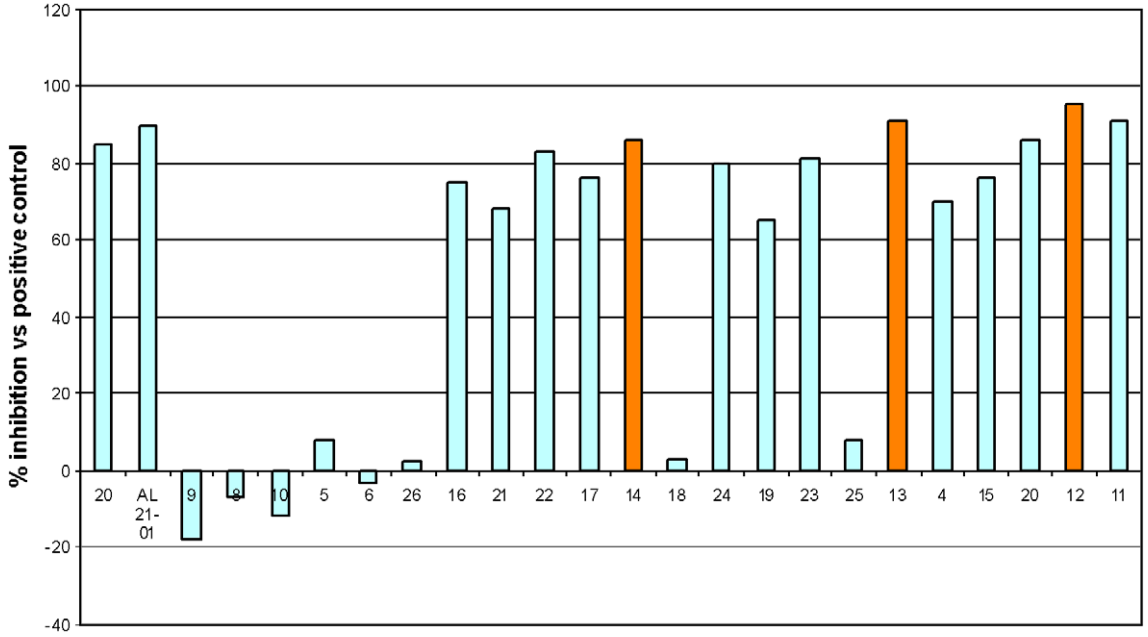


Figure 8. Screening of compounds **4**, **5** and **6**, **9**–**10**, **11**–**13**, **14**, **15**–**19**, **20**–**26** at 10 μM; **20** and AL21-01 are reference compounds.

Table 4. Screening at 0.1, 1, and 10 μM for selected compounds. **20** and AL21-01 are references

	% Inhibition		
	10^{-7} M	10^{-6} M	10^{-5} M
20	4	23	85
AL21-01	8	75	92
16	14	27	75
21	0	30	68
22	12	39	83
23	8	27	86
24	5	19	80
14	25	61	81
13	14	79	91
4	0	25	70

inhibition >50% at 1 μM, comparable with that obtained for AL21-01 (Table 4).

From those preliminary results we could confirm some of our hypothesis. First of all the presence of a reactive electrophilic group seems to be necessary to react with the cysteine of the active site of 3CP. Halo substituted ketones are reactive, especially Br and Cl mono substituted. Also, results showed that the best compounds, **13** and **14**, have a CH_2Cl reactive group which was hypothesised to be the best compromise between reactivity and tendency to hydration. Also, the presence of the meta-amide and of a meta-aromatic group looks quite interesting. The aromatic- not too bulky-group should be able to fill the S2 pocket of the protease thus increasing activity and the amide should contribute to form hydrogen bonds within the active site of protease. The type of aromatic group apparently did not make any distinction towards activity: compounds **13** and **14** contain, respectively, a phenyl and a naphthyl group and show equal activity. The nature of the linker seems to be important for inhibition: almost all of the best molecules contain a methylene group linking the two aromatic portions. We can hypothesize that presence of hydrogen bond interactions in proximity of the S2 pocket is not favorable for inhibition activity. An interesting result is the high percent inhibition of compound **4** which will be further studied.

We decided to test few representative compounds, **12**, **13**, **14** and **20**, for cytotoxicity on HeLa cells and for anti-viral activity against HRV-14. As expected, compounds **13** and **14** showed high cytotoxicity with $MNTD_{50}$ (maximum non-toxic dose which reduces 50% of cells) = 0.75 μg/ml and inhibitory activity on HRV-14 at doses higher than the toxic ones (ID_{50} > 0.75 μg/ml). Same result gave compound **12** with $MNTD_{50}$ = 3.12 μg/ml and activity on HRV-14 at higher doses. However, compound **20** proved to be quite interesting having an $MNTD_{50}$ > 100 μg/ml and ID_{50} = 25 μg/ml (IP > 4). Compound **20** was also tested on more cellular lines (VERO, MDCK) and exhibited low toxicity and good anti-viral activity against HSV2 (Herpes Simplex-2) and WSN influenza A virus at doses lower than the toxic ones (data not shown).

Results so far obtained show the higher cytotoxicity of CH_2Cl group over CH_2Br group, which prevented us from performing all the scheduled tests on HeLa cells. However, a good nucleophile keeps the inhibitory activity already reported from Reich et al.[21] 1,3,5 trisubstituted benzamides have again comparable activity on HRV-14 but we need to investigate in more detail how different groups that fill the S2 pocket can enhance activity.

5. Virtual docking of compounds 13 and 20

We decided to explore the orientations of compounds **13** and **20** by virtual docking in the binding site of HRV-2. This is shown in Figure 9A and B.

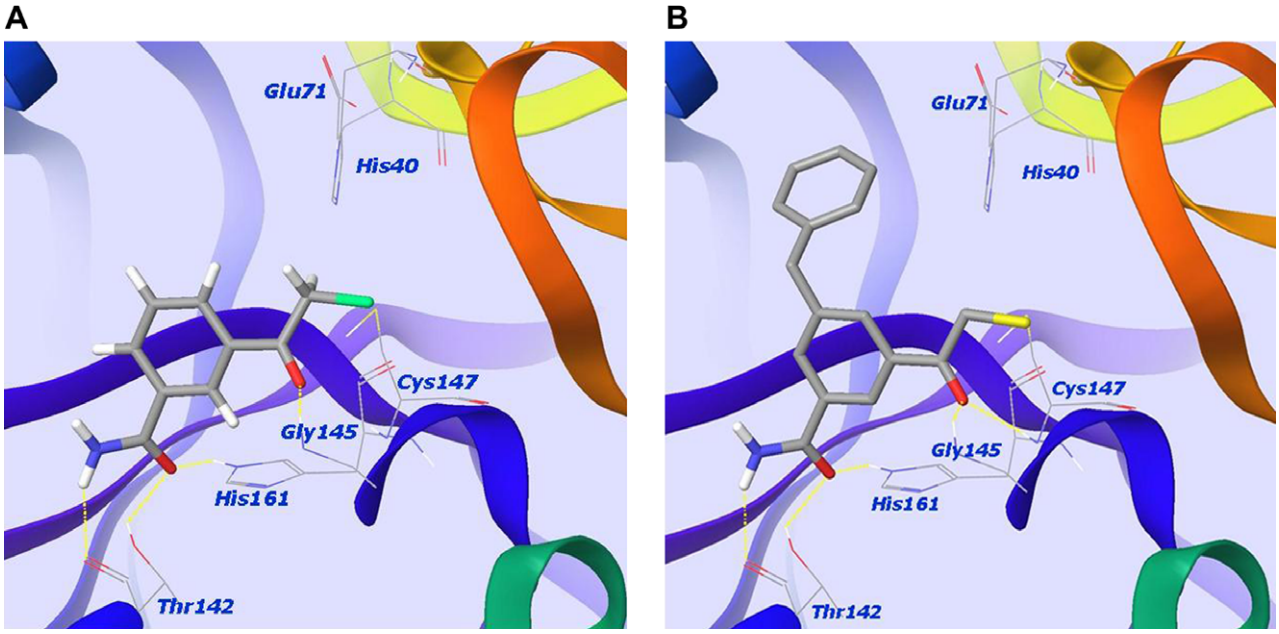


Figure 9. Representation of co-crystal structures of compounds **20** (A) and **13** (B) with HRV-2 3CP. Hydrogen bonds are represented in yellow.

Minimum energy pose of both compounds corresponds to the orientation found in AG7088 co-crystallized with HRV-2 3CP (Fig. 2) and matches the orientation of Michael-acceptor benzamides[21] where the carboxamide and the electrophile group are away from each other. The amidic group of **13** and **20** lies in the S1 pocket and it mimics the lactamic group of AG7088 as it is stabilized by hydrogen bonds with Thr142 and His161. Orientation of compound **20** is further stabilized by formation of a hydrogen bond between the carboxamide oxygen and Gly145. Also, the reactive group (aloketone) of both compounds is placed in the S3 pocket towards the catalytic site of 3CP.

Compound **13** is even more stabilized by additional hydrogen bond between the carboxamide oxygen and Cys147. It also contains a meta benzyl substituent, which lacks in compound **20**, that leans forward S2 pocket. This fact probably explains the better inhibitory activity of compound **13** over compound **20** and underlies the need for additional interaction than 1,3 disubstituted benzamides do. However, we do not see any chemical interaction between the benzyl group of compound **13** and 3CP and this fact suggests us to consider in more detail other possible groups that could interact somehow with 3CP.

6. Conclusions

In this study we demonstrated the ability of some 1,3 disubstituted and 1,3,5 trisubstituted benzamides to inhibit HRV-14 3CP. We supported further evidence for the need of an electrophilic reactive group, a primary amide and an aromatic portion to increase the activity. We also have encouraging results from 3,4-isoindolinone-like structures.

Results so far obtained look quite promising and need a better understanding of the mechanisms involved in the reactivity of 3CP in order to have more detailed SAR studies. Also, compounds here reported need further investigations concerning cellular activity and, more important, cytotoxicity.

7. Experimental

7.1. Molecular modelling

The initial minimization was performed using the MacroModel program (Schrödinger, LLC, New York, NY). Inhibitor and Cys 147 residue were fully flexible while other residues were harmonically constrained to their crystallographic positions. The employed force field was OPLS-AA choosing an electrostatic treatment distance-dependent with the dielectric constant set to 4R.

Docking was performed using GLIDE[26] program (Schrödinger, LLC, New York, NY). Structure was prepared using the default Protein Preparation. Grids for molecular docking were calculated employing 0.8, 0.9, and 1.0.Van der Waals scaling parameters. Known inhibitors of 3CP[21] were employed to establish the best combination of scaling parameters to be used. Virtual library 3D structures were generated using the LigPrep software, which generates a minimized conformation of each ligand and multiply protonation/tautomerization states when appropriate.

7.2. Biology

7.2.1. Enzymatic assay. A colorimetric assay was performed, based on chromogen substrate (a *p*-nitroaniline conjugated peptide) cleavage by protease. After hydrolysis the enzyme releases yellow-colored free *p*-nitroaniline, whose absorbance can be measured at 405 nm against a blank where either substrate or enzyme is not included in the reaction mixture. Test was performed on 96-well plates containing 200 μl/well reaction mixture. Reaction mixture includes recombinant HRV14-3C protease at 0.7 μM, protease substrate (EALFQ-pNA) and compounds at 10 μM final concentration (1% DMSO final concentration) in the reaction buffer (25 mM Hepes, pH 7.5, 150 mM NaCl, 0.12 mM DTT and 1 mM EDTA). Reaction was initiated by enzyme addiction and absorbance was read at 405 nm at time 0 and after 60 minutes of incubation at 30 °C, using a spectrophotometer (Victor II, Wallac). Leupeptin 1 mM was used as inhibition control. Blank samples were prepared for each compound in order to check for absorbance interferences.

Percent inhibition was calculated for each sample with respect to positive control in the same plate (mean of duplicate), which represented 100% of reaction. Mean value of inhibition was then calculated between compound replicates. Data are expressed as means ± SDV.

7.2.2. Cytotoxicity and anti-viral activity. Test compounds were dissolved in DMSO at a concentration of 25 mg/ml and kept in refrigerator until use.

Cytotoxic and anti-viral activities were examined in HeLa cells (Ohio strain, human adenocarcinoma) incubated at 33 °C in a 3% CO_2 atmosphere. They were cultured in DMEM with 1% non-essential aminoacids and 10% foetal calf serum (Gibco Labs. Inc.). Anti-viral activity was assayed on common cold viruses HRV-14 (type A) and HRV-39 (type B) obtained from ATCC (USA).

Cytotoxic activity was determined on HeLa cells cultured on 24 multiwell plates at a density of about 10^4/ cells per ml and incubated in the presence of decreasing concentrations of the tested compounds. After 5 days the cytotoxic effect of the compounds was checked by the incorporation of a vital dye (neutral red). The maximum non-toxic dose ($MNTD_{50}$), which is the dose that inhibits less than 50% cell replication compared to the control, was calculated by regression analysis. All the experiments were done in triplicate.

The Rhinovirus inhibition was evaluated by the analysis of the viral replication cycle on cell monolayers and subsequent titration under nutrient agar. HeLa cell mono-

layers in 24-well plates were infected with the viruses at a multiplicity of infection (MOI) of 0.1. Then decreasing amounts of the test compounds were added and after 24 h of incubation the cells underwent a cycle of freeze-thawing. The supernatant was centrifuged and tested on cell monolayers under 2% nutrient agar to detect the number of viral infectious units produced staining with neutral red and counting the visible viral plaques.

The addition of test compounds at various times before (−1 h and t0) and after (from +1 to +6 h) viral infection was used for determining the inhibited virus cycle step. Sub-confluent HeLa cells in 24-well plates were infected by the Rhinoviruses at a MOI of 0.1 for 1 h. After 48 h of incubation, the cytopathic effect was checked, the plates were freeze-thawed and the viral particles produced were counted under nutrient agar.

The neutralizing activity of the test compounds on viral infectivity was studied on HRV-14. A suspension of the test virus with about 106 Infectious Units (IU) per ml was challenged with decreasing doses of the compounds to be assayed. After 1 h of contact at 37 °C, the viral suspensions were titrated on HeLa cell monolayers under nutrient agar to determine the remained live viruses.

Cytotoxicity on VERO and MDCK cultured cells was determined on 24 multiwell plates in a similar manner. Anti-viral activity was evaluated after 48 h of incubation at 37 °C and 5% CO_2.

7.3. Chemistry

All reactions were performed under nitrogen atmosphere unless otherwise noted. Reagents and starting materials were obtained from commercial sources and used as received. Organic solutions were dried over Na_2SO_4. ^{1}H NMR were recorded on a Brucker Avance 300 instrument and chemical shifts (δ) are reported relative to TMS and/or referenced to the solvent in which they were measured. Combined HPLC-UV analysis were recorded using a Waters Alliance 2795 Separations Module and Waters 2487 Dual λ Absorbance Detector. LC/MS were recorded on API 365 Applied Biosystem equipped with ionSPRAY interface. Elemental analysis was performed using a Carlo Erba EA1108 instrument.

7.3.1. 6-Bromoisoindolin-1-one[39] (29). Dry $AlCl_3$ (2.5 g, 18.7 mmol) was placed in a three bottom flask under N_2 and a solution of isoindolin-1-one (1 g, 7.5 mmol) in dry 1,2-dichloroethane (15 mL) was added. Br_2 (0.50 mL, 9.7 mmol) was then added dropwise and the mixture was refluxed for 15 h. After cooling to rt, the excess of bromine was destroyed with an aqueous solution of sodium thiosulfate. The mixture was washed with ethyl acetate and the organic layer dried and concentrated. Chromatography on silica gel (10% ethyl acetate/hexane) gave **29** (0.640 g, 3.02 mmol, 45%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.91 (1H, m), 7.73 (1H, dd, J = 8.1, 1.8 Hz), 7.47 (1H, d, J = 8.1 Hz), 4.41 (2H, s); MS (ESI) *m/e* 212.0 [(M+H$^+$)], 214 [(M+2+H$^+$)].

7.3.2. 6-Acetylisoindolin1-one (30). To a solution of **29** (0.5 g, 2.34 mmol) and ethyl 1-(trimethylstannyl)vinyl ether (0.827 g, 3.52 mmol) in NMP (10 mL), $Pd(PPh_3)_4$ (0.136 g, 0.12 mmol) was added and the mixture was refluxed for 24 h. The reaction mixture was quenched by addition of saturated aqueous ammonium chloride and extracted with ethyl acetate, dried and concentrated. The crude product was dissolved in THF (5 mL) and HCl 2 N (0.6 mL) was added. The mixture was stirred at r.t. for 30 min and the solution was concentrated and filtered to obtain a white solid **30** (0.205 g, 1.17 mmol, 50%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.41 (1H, s), 8.22 (1H, dd, J = 7.9, 1.6 Hz), 7.57 (1H, d, J = 7.9 Hz), 6.60 (1H, s broad) 4.52 (2H, s), 2.66 (3H, s). Anal. Calcd for $C_{10}H_9NO_2$: C, 68.56; H, 5.18; N, 8.00. Found: C, 68.62; H, 5.12; N, 8.08.

7.3.3. 6-(Bromoacetyl)isoindolyn-1-one (4). Br_2 (0.058 mL, 1.14 mmol) was added dropwise to a solution of **30** (0.2 g, 1.14 mmol) in chloroform (10 mL). The reaction mixture was stirred at 70 °C for 20 min, cooled to rt and filtered to give **4** (0.22 g, 0.87 mmol, 76%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 4.49 (2 H, s) 5.02 (2 H, s) 7.76 (1 H, dd, J = 8.04, 0.73 Hz) 8.20 (1 H, dd, J = 7.97, 1.68 Hz) 8.27 (1 H, d, J = 1.02 Hz) 8.75 (1 H, s broad); MS (ESI) *m/e* 254.0 [(M+H$^+$)], 256 [(M+2+H$^+$)]. HPLC t_R 13.7 min; purity 93% Synergy hidro-RP 250X4.6 mm 4 μm, gradient elution of 10–40% CH_3CN (0.01% TFA)/water(0.01% TFA) for 30 min, λ = 218 nm. Anal. Calcd for $C_{10}H_8BrNO_2$: C, 47.27; H, 3.17; N, 5.51. Found: C, 46.82; H, 2.92; N, 5.22.

7.3.4. 4-Bromoisoindolin-1-one (33). NH_3/MeOH was added dropwise to a solution of methyl 3-bromo-2-(bromomethyl)benzoate (0.735 g, 2.38 mmol) in MeOH (7 mL) and stirred at rt until the formation of a white precipitate. The mixture was filtered and the solid was washed with Et_2O and dried to give the desired product **33** (0.479 g, 2.26 mmol, 95%). ^{1}H NMR (500 MHz, CD_3OD) δ: 7.78 (2H, d, J = 7.7 Hz), 7.46 (1H, t, J = 7.7 Hz), 4.38 (2H, s); MS (ESI) *m/e* 212.0 [(M+H$^+$)], 214 [(M+2+H$^+$)].

7.3.5. 4-Acetylisoindolil-1-one (34). Prepared following the procedure described for **30**. (0.205 g, 1.17 mmol, 50%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.09 (1H, d, J = 7.5 Hz), 8.07 (1H, d, J = 7.6 Hz), 7.62 (1H, t, J = 7.6 Hz), 6.88 (1H, s broad) 4.79 (2H, s), 2.66 (3H, s). Anal. Calcd for $C_{10}H_9NO_2$: C, 68.56; H, 5.18; N, 8.00. Found: C, 68.64; H, 5.14; N, 8.11.

7.3.6. 4-(Bromoacetyl)isoindolin-1-one (5). Prepared following the procedure described for **4**. (0.045 g, 0.18 mmol, 50%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.75 (s, 1 H), 8.31 (dd, J = 7.75, 1.02 Hz, 1 H), 7.97 (dd, J = 7.60, 0.88 Hz, 1 H), 7.70 (t, J = 7.60 Hz, 1 H), 5.01 (s, 2 H), 4.62 (s, 2 H). MS (ESI) *m/e* 254.0 [(M+H$^+$)], 256 [(M+2+H$^+$)]. HPLC t_R 10.6 min; purity 90% PKB-100 250 × 4.6 mm 5 μm, gradient elution of 5–55% CH_3CN/CH_3COONH_4 10 mM for 20 min, λ = 250 nm. Anal. Calcd for $C_{10}H_8BrNO_2$: C, 47.27; H, 3.17; N, 5.51. Found: C, 47.02; H, 3.03; N, 5.42.

7.3.7. Ethyl 3-amino-5-[1-(hydroxyamino)ethyl]benzoate (36). Ethyl 3,5-dinitrobenzoate (15 g, 62.5 mmol), Pd/C 10% (0.685 g, 0.6 mmol) and Et_3N (39.2 mL) were placed in a three-bottom flask equipped with a condenser. When the mixture started to reflux formic acid (10.1 mL, 269 mmol) was added dropwise. The solution was stirred for 20′. After being cooled at room temperature the reaction mixture was diluted with dichloromethane and filtered off Celite. The organic layer was washed with water, dried and concentrated. The crude product was used for the next reaction without further purification.

Ethyl 3-amino-5-nitrobenzoate (5.54 g, 26.4 mmol) was dissolved in water (33 mL) and HCl 37% (2.1 mL). The mixture was stirred at 0 °C and sodium nitrite (2 g, 29 mmol) was added. The reaction mixture was stirred for 20 min more at 0 °C. In the meanwhile acetaldehyde oxime (3.12 g, 52.8 mmol) was dissolved in 5 mL of water and $CuSO_4$ pentahydrate (1.98 g, 7.92 mmol) and sodium acetate trihydrate (19.1 g, 140 mmol) were added. The solution was cooled to 0–5 °C and the diazonium salt was added. The reaction mixture was stirred at 10–15 °C for 1.5 h. HCl 37% (12.1 mL) was added and the solution was refluxed for 2 h. After being cooled at room temperature the reaction mixture was extracted with ethyl acetate and concentrated. The crude product was used for the next reaction without further purification.

To a solution of ethyl 3-[(1-*N*-hydroxyethanimidoyl)]-5-nitrobenzoate (1.8 g, 7.1 mmol) in abs EtOH (20 mL) $SnCl_2 * H_2O$ (8.5 g, 37.7 mmol) was added. The solution was stirred at 70 °C for 1 h and poured in to a $NaHCO_3$ ice bath. The precipitate was filtered off and the alkaline solution was extracted with ethyl acetate. The organic layer was dried and concentrated. The residue was purified by flash chromatography on silica gel (chloroform/methanol 95/5) to obtain **36** (1.3 g, 5.8 mmol, 82%). GC–MS *m/z* 222.1 (8) [M^+], 206.2 (38), 177.1 (61), 135.1 (100). Anal. Calcd for $C_{11}H_{14}N_2O_3$: C, 59.45; H, 6.35; N, 12.60. Found: C, 59.22; H, 6.29; N, 12.58.

7.3.8. 3-(Benzylamino)-5-[1-*N*-hydroxyethanimidoyl]benzamide (6). Compound **36** (1.3 g, 5.8 mmol) was placed in a two bottom flask and 40 mL of NH_3 32% were added. The reaction mixture was stirred for 48 h. The solution was diluted with water and extracted with dichloromethane. The organic layer was dried and concentrated. The crude residue was used for the next reaction without further purification.

3-amino-5-[1-*N*-hydroxyethanimidoyl]benzamide (0.35 g, 1.81 mmol), K_2CO_3 (0.27 g, 1.95 mmol), *i*-BuOH (10 mL) and EtOH (10 mL) were placed in a three bottom flask equipped with a condenser and a magnetic stirrer. Benzyl chloride (0.23 mL, 2 mmol) was added and the mixture was refluxed o.n. After being cooled at room temperature the solution was filtered and the solvent was evaporated in vacuo. The residue was purified by flash chromatography on silica gel (chloroform/methanol 95/5) to obtain **6** (0.175 g, 0.6 mmol, 34%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 9.77 (br s, 3H), 8.28 (br s, 1 H), 8.10 (t, J = 1.37 Hz, 1 H), 7.84 (ddd, J = 5.49, 1.92, 1.74 Hz, 2 H), 7.57 (br s, 1 H), 7.25–7.47 (m, 5 H), 5.25 (s, 2 H), 2.29 (s, 3 H). MS (ESI) *m/e* 284.2 [($M+H^+$)]. HPLC t_R 15.6 min; purity 92% pkb-100 254 × 4.6 mm 5 μm, gradient elution of 15–45% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 minutes, λ = 206 nm. Anal. Calcd for $C_{16}H_{17}N_3O_2$: C, 67.83; H, 6.05; N, 14.83. Found: C, 67.23; H, 5.88; N, 14.66.

7.3.9. 3-(Trifluoroacetyl)benzonitrile (38). 3-Cyanobenzoic acid (4.87 g, 33 mmol) dissolved in dichloromethane (35 mL), MeOH (4 mL) and DMAP (3.22 g, 26 mmol) were placed in a two bottom flask The solution was stirred at 0 °C and then DCC (7.5 g, 36.4 mmol) was added in 30′. The reaction mixture was stirred at 0 °C for 15′ and was then warmed to rt and stirred for 4 h. The precipitate was filtered off and the filtrate was washed with HCl 0.5 N, $NaHCO_3$ and NaCl. The organic layer was dried and concentrated to obtain methyl 3-cyanobenzoate (3.9 g, 24.2 mmol, 73%), which was used for the next reaction without further purification.

Methyl 3-cyanobenzoate (1.6 g, 10 mmol) was placed in a three bottom flask under N_2 and trimethylsilyil trifluoromethane (1.85 mL, 12.5 mmol) in dry CH_2Cl_2 (25 mL) and dry CsF (0.076 g, 0.5 mmol) were added. The solution was stirred at rt for 2;days. HCl 6 N (10 mL) was added and the reaction mixture was stirred for 5 h. The mixture was separated and the water was extracted with DCM. The organic layer was washed with NaCl, dried and concentrated. The residue was purified by flash chromatography on silica gel (ethyl acetate/hexane 1/9) to obtain **38** (1 g, 5 mmol, 50%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.39–8.43 (m, 1 H), 8.28–8.35 (m, 1 H), 7.85–7.97 (m, 2 H). GC–MS *m/z* 199 (5.3) [M^+], 130.1 (100), 102.1 (92), 75 (58).

7.3.10. 3-(Trifluoroacetyl)benzamide (7). 38 (1 g, 5.02 mmol), H_2O_2 30% (1.8 mL, 17.5 mmol) and EtOH 95° (5 mL) were placed in a three bottom flask equipped with a condenser and a magnetic stirrer. The resulting mixture was stirred at 50 °C and NaOH 6 N (0.21 mL, 1.26 mmol) was added. The solution was stirred for 21 h and, after that, the reaction mixture was neutralized with HCl 6 N. The solution was concentrated, diluted with water and extracted with DCM. The white precipitated obtained was filtered off to give **7** (0.52 g, 2.39 mmol, 47%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.52 (s, 1 H), 8.33 (dt, J = 7.76, 1.40 Hz, 1 H), 8.29 (br s, 1 H), 8.18 (dt, J = 7.84, 0.87 Hz, 1 H), 7.78 (t, J = 7.84 Hz, 1 H), 7.65 (br s, 1 H). GC–MS *m/z* 217.1 (23) [M^+], 201.0 (38), 173.0 (40), 148.0 (100). HPLC t_R 8.5 min; purity 99.9% pkb-100 254 × 4.6 mm 5 μm, gradient elution of 10–30% CH_3CN/H_2O for 20 min, λ = 224 nm. Anal. Calcd for $C_9H_6F_3NO_2$: C, 49.78; H, 2.79; N, 6.45. Found: C, 49.79; H, 2.61; N, 6.45.

7.3.11. Methyl 3-bromo-5-{2,2-difluoro-1-[(2-methoxyethoxy)methoxy]ethenyl}benzoate (40). To a solution of 3-bromo-5-iodobenzoic acid (1 g, 3.06 mmol) in dry

MeOH (5 mL) dry HCl (0.306 mL of a 1 M solution in diethyl ether) was added. The reaction mixture was stirred at room temperature for 24 h. The solvent was evaporated in vacuo. Chromatography on silica gel (20% ethyl acetate/petroleum ether) gave the ester intermediate (0.930 g, 2.72 mmol, 89%).

In a reaction vessel equipped with a condenser and a magnetic stirrer $Pd(OAc)_2$ (25 mg, 0.11 mmol), CuI (172 mg, 0.932 mmol) and PPh_3 (108 mg, 0.41 mmol) were placed under a stream of argon. Dry DMF (5 mL) and the aryl bromide intermediate (1.4 g, 4.106 mmol) were then added. The resulting mixture was stirred at 60 °C and the stannane $F_2C{=}C(OMEM)SnBu_3$ was added. The mixture was stirred at 60 °C for 16 h. After being cooled at room temperature the reaction mixture was quenched with a saturated aqueous NH_4Cl solution and the resulting mixture was extracted with AcOEt. The organic extract was washed with water, dried, filtered through Celite and the filtrate was concentrated under reduced pressure. The residue was purified by chromatography on silica gel (20% ethyl acetate/petroleum ether) obtaining **40** (1.016 g, 2.66 mmol, 67%). ^{1}H NMR (200 MHz, $CDCl_3$) δ ppm 8.18 (2 H, s) 8.12 (2 H, s) 7.85 (1 H, s) 4.91 (1 H, s) 3.97 (3 H, s) 3.93–3.85 (2 H, m) 3.62–3.51 (2 H, m) 3.41 (3 H, s). Alal. Calcd for $C_{15}H_{19}BrF_2O_5$: C, 45.36; H, 4.82. Found: C, 45,22; H, 4.82.

7.3.12. 3-Benzyl-5-(difluoroacetyl)benzamide (9). Dry $Pd(PPh_3)_4$ (0.151 g, 0.131 mmol) was placed in a three bottom flask under N_2. Dry THF (15 mL), **40** (1 g, 2.62 mmol) and BnZnBr (13 mL, 6.5 mmol) were added. The resulting mixture was stirred at 70 °C. The degree of completion of the reaction mixture was evaluated by GC analyses of a sample of the crude reaction mixture. After being cooled at room temperature the reaction was quenched with a saturated aqueous NH_4Cl solution and the resulting mixture was extracted with AcOEt. The organic extract was washed with water, dried, filtered through Celite and the filtrate was concentrated under reduced pressure. The residue was purified by chromatography on silica gel (20% ethyl acetate/petroleum ether) obtaining the benzylated compound.

To a solution of the benzylated compound (0.667 g, 1.7 mol) in MeOH at 0 °C Me_3SiCl (0.4 mL, 3 mmol) was added and the mixture was stirred overnight. The solvent was evaporated and the crude product was used for the next step without further purification. The residue was dissolved in KOH/EtOH 10% (10 mL) and the mixture was stirred until the TLC showed the end of the reaction. The solvent was evaporated, the crude residue was dissolved in water and the solution was extracted with ethyl acetate. The organic layer was neutralized with HCl 3 N and the aqueous phase was extracted with ethyl acetate; the organic layer was dried and evaporated in vacuo to obtain the acid (0350 g, 1.2 mmol), that was used for the next reaction without further purification.

3-Benzyl-5-(difluoroacetyl)benzoic acid (0.350 g, 1.21 mmol) was dissolved in $SOCl_2$ (35 mL) and the mixture was stirred at 80 °C. The degree of completion of the reaction was evaluated by IR spectroscopy of a sample of the crude reaction mixture. After being cooled at room temperature $SOCl_2$ was evaporated in *vacuo* and the crude residue was dissolved in dry THF. NH3(g) was bubbled for 1 h into the solution. Formation of amide bond was evaluated by IR spectroscopy. The solvent was evaporated in vacuo and the residue was purified by chromatography on silica gel (ethyl acetate). Crystallization by ethyl acetate/hexane yield **9** (0.280 g, 0.96 mmol, 80%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.34 (s, 1 H), 8.19 (br. s., 1 H), 8.13 (s, 1 H), 8.04 (s, 1 H), 7.56 (br s, 1 H), 6.93–7.40 (m, 6 H), 4.11 (s, 2 H). GC–MS (70 eV; rel Int): 289 [M^+], 238, 165, 91. HPLC t_R 6.5 min; purity 95% Platinum 100 150 × 4.6 mm 3 μm, gradient elution 5–25% CH_3CN/CH_3COONH_4 for 20 min, λ = 207 nm. Anal. Calcd for $C_{16}H_{13}F_2NO_2$: C, 66.43; H, 4.53; N, 4.84. Found: C, 66.12; H, 4.30; N, 4.98.

7.3.13. 3-(Difluoroacetyl)benzamide (8). Prepared following the procedure described for **9** from 3-iodobenzoic acid as starting material (0.280 g, 1.4 mmol, 70%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.50 (s, 1H), 8.11–8.28 (m, 3 H), 7.72 (t, J = 7.75 Hz, 1 H), 7.59 (br s, 1 H), 7.20 (t, J = 52.50 Hz, 1 H). MS (ESI) *m/e* 198 [(M^-)]. HPLC t_R 8.6 min; purity 98% Pentium 100 150 mm 3 μm, gradient elution 5–45% CH_3CN/CH_3COONH_4 10 mM for 20 min, λ = 226 nm. Anal. Calcd for $C_9H_7F_2NO_2$: C, 54.28; H, 3.54; N, 7.03. Found: C, 53.95; H, 3.54; N, 6.76.

7.3.14. Methyl-3-{2,2-difluoro-1-[(2-methoxyethoxy)methoxy]ethenyl}-5-{[(trifluoromethyl)sulfonyl]oxy}benzoate (43). Methyl 3,5-dihydroxybenzoate (5 g, 29.7 mmol) was placed in a three bottom flask under N_2 and was dissolved in dry dichloromethane (30 mL). Et_3N (1.79 g, 178.2 mmol) was added. The mixture was stirred for 5′ at rt and then was cooled at 0 °C. Fifteen millilitres of a solution of trifluoromethansulfonic anhydride in CH_2Cl_2 was added dropwise. The mixture was stirred for 4 h at rt, then was quenched with water and extracted with dichloromethane. The organic layer was dried and the solvent was evaporated in vacuo. The residue was purified by chromatography on silica gel (ethyl acetate/petroleum ether 1/9) to obtain methyl 3,5-bis(trifluoromethyl)sulfonyloxy benzoate (10.3 g, 23.8 mmol, 80%).

CuI (0.044 g, 0.231 mmol), $Pd(dba)_3$ (0.043 g, 0.046 mmol), $AsPh_3$ (0.057 g, 0.185 mmol), LiCl (0.294 g, 6.9 mmol) and the intermediate ester (1 g, 2.3 mmol) were placed in a three bottom flask under N_2. NMP (8 mL) and the stannane (1.47 g, 3.18 mmol) were added and the mixture was stirred at 60 °C for 2 h. After being cooled to room temperature the reaction mixture was quenched with water and extracted with ethyl acetate; the organic layer was dried and concentrated in vacuo. The residue was purified by chromatography on silica gel (ethyl acetate/petroleum ether 1/1) to obtain **43** (0.5 g, 1.1 mmol, 50%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.18 (1H, s), 7.84 (1H, s), 7.57 (1H, s), 4.88 (2H, s) 3.97 (3H, s), 3.90-3.80 (2H, m), 3.50–3.40 (2H, m), 3.55 (3H, s). GC-MS (70 eV; rel Int): 419(2) [M^+-31], 348 (8), 311 (13), 89 (96), 59 (100). Anal. Calcd for $C_{16}H_{19}F_5O_8S$: C, 41.21; H, 4.11. Found: C, 41.18; H, 4.11.

7.3.15. Methyl 3-benzamido-5-{2,2-difluoro-1-[(2-methoxyethoxy)methoxy]ethenyl}benzoate (44). $Pd(AcO)_2$ (0.021 g, 0.022 mmol), Xantphos (0.039 g, 0.066 mmol), benzamide (0.175 g, 1.44 mmol) and K_2CO_3 (0.215 g, 1.55 mmol) were placed in a three bottom flask under N_2. 1.5 mL of a 1 M solution of **43** in dioxane was added and the reaction mixture was stirred for 20 h. The mixture was diluted with dichloromethane, filtered through Celite and concentrated. The residue was purified by chromatography on silica gel (ethyl acetate/petroleum ether 1:1) to obtain **44** (0.150 g, 0.35 mmol, 33%). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 8.21 (2H, s), 8.05 (1H, s), 7.88–7.70 (3H, m), 7.53 (1H, t, J = 8.3 Hz) 7.45 (2H, t, J = 7.7 Hz). Anal. Calcd for $C_{22}H_{25}F_2NO_6$: C, 60.41; H, 5.76; N, 3.20. Found: 60.55; H, 5.81; N, 3.25.

7.3.16. 3-(Difluoroacetyl)-5-[(phenylcarbonyl)amino]benzamide (10). Prepared following the procedure described for **9** from**44** as starting material (0.055 g, 0.17 mmol, 49%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 10.65 (s, 1H), 8.64 (d, J = 1.65 Hz, 2 H), 8.24 (s, 1 H), 8.20 (br s, 1 H), 7.98–8.07 (m, 2 H), 7.48–7.68 (m, 4 H), 7.15 (t, J = 52.50 Hz, 1 H). MS (ESI) *m/e* 317.1 [(M^-)], 349.1 [(M^-+MeOH)]. HPLC t_R 4.6 min; purity 90% Platinum 100 150 mm 3 μm, gradient elution of 2–18% CH_3CN/CH_3COONH_4 10 mM for 16 min, λ = 205 nm. Anal. Calcd for $C_{16}H_{12}F_2N_2O_3$: C, 60.38; H, 3.80; N, 8.80. Found: C, 60.21, H, 3.70, N, 8.73.

7.3.17. 3-Acetylbenzamide (46). Prepared following the procedure described for **7** (1.5 g, 9.2 mmol, 67%) from 3-acetylbenzonitrile as starting material. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 2.67 (3H, s), 5.93 (1 H, br s), 6.30 (1 H, br s), 7.58 (1 H, t, J = 7.8 Hz), 8.06 (1 H, dt, J = 7.8, 1.5 Hz), 8.12 (1 H, dt, J = 7.8, 1.5 Hz), 8.40 (1 H, t, J = 1.5 Hz); MS (EI) *m/z* 164.0671 [M^+], 148 (100).

7.3.18. 3-(Dibromoacetyl)benzamide (15), 3-(bromoacetyl)benzamide (20). Br_2 (0.31 mL, 6.1 mmol) was added to a solution of **46** (1 g, 6.1 mmol) in AcOH (90 mL). The reaction mixture was stirred at 60 °C for 4 h. The solvent was evaporated in vacuo and the crude residue was purified by chromatography on silica gel (hexane/ethyl acetate 2:8). Two products were isolated **15** (0.3 g, 0.93 mmol, 15%) and **20** (0.8 g, 3.3 mmol, 54%). **15** ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.53 (t, J = 1.75 Hz, 1H), 8.15–8.26 (m, 2 H), 8.14 (br s, 1 H), 7.86 (s, 1 H), 7.57 (br s, 1 H) 7.68 (t, J = 7.75 Hz, 1 H). GC–MS *m/z* 163.1 (1) [M^+−2Br], 148.0 (100). HPLC t_R 21.3 min; purity 92% 18-DB 254 × 4.6 mm 5 μm, gradient elution of 10–45% CH_3CN/H_2O (HCOOH 0.1%) for 35 min, λ = 219 nm. Anal. Calcd for $C_9H_7Br_2NO_2$: C, 33.68; H, 2.20; N, 4.36. Found: C, 33.45; H, 2.11; N, 4.26. **20** ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.48 (t, J = 1.53 Hz, 1 H), 8.10–8.19 (m, 3 H), 7.65 (t, J = 7.82 Hz, 1 H), 7.54 (br s, 1 H), 4.97 (s, 1 H). GC–MS *m/z* 241.0 (2) [M^+] 163.1 (13) [M^+−Br], 147.9 (100). HPLC t_R 12.6 min; purity 91% 18-DB 254 × 4.6 mm 5 μm, gradient elution of 10–45% CH_3CN/H_2O (HCOOH 0.1%) for 35 min, λ = 219 nm. Anal. Calcd for $C_9H_8BrNO_2$: C, 44.66; H, 3.33; N, 5.79. Found: 44.52; H, 3.32; N, 5.71.

7.3.19. 3-(Chloroacetyl)benzamide (12), 3-(dichloroacetyl)benzamide (11). $(CH_3)_3SiCl$ (7.95 mL, 18 mmol) was added to a solution of 3-acetylbenzonitrile (1.45 g, 10 mmol) and MnO_2 (1.56 g, 15 mmol) in AcOH (15 mL) and the mixture was stirred at 0 °C o.n. The solution was poured into water and extracted with a Et_2O/eptane 1:1. The organic layer was washed with NaOH 0.025 M, dried and concentrated. The mixture of mono and dichloro compounds (0.741 g) was used for the next reaction without further purification.

The mixture was placed in a three bottom flask equipped with a condenser and H_2SO_4 (2 mL) was added. The mixture was stirred at 50 °C for 2 h. The solution was diluted with water and extracted with chloroform, dried and concentrated. The residue was purified by flash chromatography on silica gel (chloroform/methanol 9:1) to obtain two isolated compounds **12** (0.14 g, 0.71 mmol) and **11** (0.262 g, 1.13 mmol).**12** ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.46 (t, J = 1.53 Hz, 1 H), 8.05–8.22 (m, 3 H), 7.58–7.71 (m, 1 H), 7.54 (br s, 1 H), 5.24 (s, 2 H). GC–MS *m*/it z 148 (100) [M^+−CH_2Cl], 130.1 (30). HPLC t_R 9.2 min; purity 75.7% pkb 100 254 × 4.6 mm 5 μm, gradient elution of 10–40% CH_3CN/H_2O for 30 min, λ = 284 nm. Anal. Calcd for $C_9H_8ClNO_2$: C, 54.70; H, 4.08; N, 7.09. Found: C, 54.79; H, 4.10; N, 7.02. **11** ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.54 (t, J = 1.65 Hz, 1 H), 8.18–8.24 (m, J = 8.03, 2.46, 1.20, 1.20 Hz, 2 H), 8.17 (br s, 1 H), 7.91 (s, 1 H), 7.70 (t, J = 7.76 Hz, 1 H), 7.59 (br s, 1 H). GC–MS *m/z* 148.1 (100) [M^+−$CHCl_2$]. HPLC t_R 17.1 min; purity 99.8% pkb 100 254 × 4.6 mm 5 μm, gradient elution of 10–40% CH_3CN/H_2O for 30 min, λ = 251 nm. Anal. Calcd for $C_9H_7Cl_2NO_2$: C, 46.58; H, 3.04; N, 6.04. Found: C, 46.43; H, 2.97; N, 5.92.

7.3.20. Methyl 3-benzyl-5-bromobenzoate (47a). HCl 37% (0.255 mL, 3.059 mmol) was added to a solution of 3-bromo-5-iodobenzoic acid (10 g, 30.5 mmol) in MeOH (200 mL). The reaction mixture was stirred at 70 °C o.n. The solution was poured into water and extracted with ethyl acetate; the organic layer was dried and concentrated to obtain the methyl 3-bromo-5-iodobenzoate. The crude residue was dissolved in 20 mL of THF and placed in a three bottom flask under N_2. $Pd(PPh_3)_4$ (0.560 g, 48.5 mmol) was added and the solution was stirred at room temperature for 5′. A solution 0.5 M of benzylzinc bromide (21.3 mL, 10.7 mmol) was added and the reaction mixture was stirred at 60 °C o.n. The solution was cooled at 0 °C and water (30 mL) and HCl 2 N (15 mL) were added. The aqueous phase was extracted with ethyl acetate; the organic layer was filtered through Celite, dried and concentrated. The residue was purified by flash chromatography on silica gel (hexane/ethyl acetate 9/1) to obtain **47a** (1.8 g, 5.8 mmol, 60%). GC–MS *m/z*: 304(77) [M^+], 165 (100). Anal. Calcd for $C_{15}H_{13}BrO_2$: C, 59.04; H, 4.29; Found: C, 59.17; H, 4.35.

7.3.21. Methyl 3-bromo-5-(5,8-dihydronaphthalen-2-ylmethyl)benzoate (47b). Prepared following the procedure described for **47a** (3 g, 8.4 mmol, 60%). ^{1}H NMR

(300 MHz, DMSO-d_6) δ ppm 7.72–7.93 (m, 7 H), 7.44–7.53 (m, 2 H), 7.41 (dd, J = 8.33, 1.75 Hz, 1 H), 4.21 (s, 2 H), 3.83 (s, 3 H). GC–MS *m/z*: 356 (44) [M^+], 215 (100), 141 (46), 107 (48). Anal. Calcd for $C_{19}H_{15}BrO_2$: C, 64.24; H, 4.26. Found: C, 64.13; H, 4.18.

7.3.22. 3-Acetyl-5-benzylbenzamide (48a). $Pd(OAc)_2$ (0.06 g, 0.27 mmol), PPh_3 (0.14 g, 0.5 mmol), butylvinylether (2.3 mL, 17.8 mmol), **47a** (1.08 g, 3.5 mmol) and Et_3N (0.592 mL, 4.3 mmol) in dry CH_3CN were placed in a two bottom flask under N_2. The reaction mixture was stirred at 100 °C o.n. After being cooled at room temperature the solution was filtered, washed with water and concentrated in vacuo. The residue was dissolved in a mixture 1:1 HCl 10%/THF (80 mL) and stirred at room temperature for 3 h. The mixture was diluted with water and extracted with ethyl acetate. The organic layer was dried and concentrated. The crude residue was used for the next step without further purification.

To a solution of the crude residue (0.45 g, 1.7 mmol) in THF (3 mL) 6 mL of NH_3 32% was added and the mixture was stirred at 80 °C for 5 h. The solution was diluted with water and extracted with ethyl acetate. The organic layer was dried and concentrated obtaining **48a** (215 g, 0.85 mmol, 50%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.21 (t, J = 1.61 Hz, 1 H), 7.88 (s, 1 H) 7.94 (s, 1 H), 7.11–7.38 (m, 5 H), 5.97 (br s, 2 H), 2.61 (s, 3 H) 4.09 (s, 2 H). MS (ESI) *m/e* 254.0 [(M+H$^+$)]. Anal. Calcd for $C_{14}H_{12}BrNO$: C, 57.95; H, 4.17; N, 4.83. Found: C, 57.98; H, 4.18; N, 4.85.

7.3.23. 3-Acetyl-5-(5,8-dihydronaphthalen-2-ylmethyl)benzamide (48b). Prepared following the procedure described for **48a** (0.5 g, 1.6 mmol, 50%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.30 (t, J = 1.61 Hz, 1H), 8.15 (br s, 1 H), 8.06 (t, J = 1.61 Hz, 1 H), 8.01 (t, J = 1.61 Hz, 1 H), 7.75–7.91 (m, 4 H), 7.34–7.54 (m, 4 H), 4.25 (s, 2 H), 2.60 (s, 3 H). MS (ESI) *m/e* 304.0 [(M+H$^+$)]. Anal. Calcd for $C_{18}H_{14}BrNO$: C, 63.55; H, 4.15; N, 4.12. Found: C, 63.59; H, 4.15; N, 4.13.

7.3.24. Dimethyl 5-acetylbenzene-1,3-dicarboxylate (50). Dimethyl 5-aminobenzene-1,3-dicarboxylate (1 g, 4.8 mmol) was dissolved in water (6 mL) and HCl 37% (0.38 mL). The mixture was stirred at 0 °C and sodium nitrite (0.36 g, 5.2 mmol) was added. The reaction mixture was stirred for an other 20 min at 0 °C. In the meanwhile acetaldehyde oxime (0.6 g, 10.2 mmol) was dissolved in 5 mL of water and $CuSO_4$ pentahydrate (0.4 g, 1.6 mmol) and sodium acetate trihydrate (3.5 g, 125.7 mmol) were added. The solution was cooled to 0–5 °C and the diazonium salt was added. The reaction mixture was stirred at 10–15 °C for 1.5 h. HCl 37% (2.2 mL) was added and the solution was refluxed for 2 h. After being cooled at room temperature the reaction mixture was extracted with ethyl acetate and concentrated. The residue was purified by flash chromatography on silica gel (hexane/ethyl acetate 8/2) to obtain **50** (0.36 g, 1.52 mmol, 42%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.87 (t, J = 1.65 Hz, 0 H), 8.78 (d, J = 1.65 Hz, 2 H), 4.00 (s, 6 H), 2.70 (s, 3 H). MS (ESI) *m/e* 237.1 [(M+H$^+$)], 194.9, 193.1.

7.3.25. 3-Acetyl-5-(phenylcarbamoyl)benzoic acid (51a). **50** (11 g, 46.6 mmol) was placed in an open flask equipped with a condenser and it was dissolved in NaOH 2 N (250 mL). The mixture was subjected to microwave irradiation at 400 W for 6 min and then at 300 W for 10 min. At the end of irradiation, the reaction mixture was cooled at room temperature and HCl 2 N was added until the complete precipitation of a solid. The precipitate was filtered and used for the next step without further purification. The compound (5 g, 24 mmol) was dissolved in DMF (130 mL) and the solution was stirred at 0 °C; HOBt (3.57 g, 26.4 mmol) and DCC (5.45 g, 26.4 mmol) were added. The reaction mixture was stirred at 0 °C for 30 min, then aniline (2.41 mL, 26.4 mmol) was added and the solution was stirred for 1.5 h. The solution was stirred for 3 h more at room temperature and filtered. The filtrated was diluted with HCl 2 N and extracted with ethyl acetate. The organic layer was neutralized with NaOH 2 N and extracted with water. HCl 2 N was added until the complete precipitation of the product. The precipitated was filtered and recrystallized from abs. EtOH obtaining **51a** (3.56 g, 12.6 mmol, 52%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 13.55 (br s, 1H), 10.60 (s, 1H), 8.74 (t, J = 1.65 Hz, 1 H), 8.71 (t, J = 1.74 Hz, 1 H), 8.61 (t, J = 1.56 Hz, 1 H), 7.74–7.84 (m, 2 H), 7.39 (t, J = 7.87 Hz, 2 H), 7.09–7.20 (m, 1 H), 2.72 (s, 3 H). MS (ESI) *m/e* 284.3 [(M+H$^+$)]. Anal. Calcd for $C_{16}H_{13}NO_4$: C, 67.84; H, 4.63; N, 4.94. Found: 67.80; H, 4.66; N, 4.99.

7.3.26. 3-Acetyl-5-(naphth-1-ylcarbamoyl)benzoic acid (51b). Prepared following the procedure described for **51a** (0.7 g, 2.1 mmol, 50%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 13.55 (br s, 1 H), 10.81 (s, 1 H), 8.86 (d, J = 1.61 Hz, 2 H), 8.65 (t, J = 1.61 Hz, 1 H), 7.96–8.04 (m, 2 H), 7.86–7.93 (m, 1 H), 7.51–7.67 (m, 4 H), 2.74 (s, 3 H). MS (ESI) *m/e* 334.4 [(M+H$^+$)]. Anal. Calcd for $C_{20}H_{15}NO_4$: C, 72.06; H, 4.54; N, 4.20. Found: 71.89; H, 4.41; N, 4.09.

7.3.27. 5-Acetyl-*N*-phenylbenzene-1,3-dicarboxamide (52a). **51a** (1.5 g, 5.3 mmol) was dissolved in thionyl chloride (15 mL) and the mixture was stirred at 50 °C for 3 h. After being cooled at room temperature the thionyl chloride was evaporated in vacuo and the crude residue was dissolved in dry THF. NH_3(g) was bubbled into the solution for 2 h. The solvent was evaporated in *vacuo* and the residue was purified by chromatography on silica gel ($CHCl_3$/MeOH 98:2) obtaining **52a** (0.6 g, 2.1 mmol, 40%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 13.55 (br s, 1 H), 10.60 (s, 1 H), 8.74 (t, J = 1.65 Hz, 1 H), 8.71 (t, J = 1.74 Hz, 1 H), 8.61 (t, J = 1.56 Hz, 1 H), 7.74–7.84 (m, 2 H), 7.39 (t, J = 7.87 Hz, 2 H), 7.09–7.20 (m, 1 H), 2.72 (s, 3 H). MS (ESI) *m/e* 284.3 [(M+H$^+$)]. Anal. Calcd for $C_{16}H_{14}N_2O_3$: C, 68.07; 5.00; N, 9.92. Found: C, 67.86; H, 4.94; N, 9.81.

7.3.28. 5-Acetyl-*N*-naphthalen-1-ylbenzene-1,3-dicarboxamide (52b). Prepared following the procedure described for **52a** (0.23 g, 0.69 mmol, 32%).^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 13.55 (br s, 1 H), 10.60 (s, 1 H), 8.74 (t, J = 1.65 Hz, 1 H), 8.71 (t, J = 1.74 Hz, 1 H),

8.61 (t, J = 1.56 Hz, 1 H), 7.82–7.91 (m, 3H), 7.45–7.53 (m, 3H), 7.42 (dd, 1H), 2.72 (s, 3 H). Anal. Calcd for $C_{20}H_{16}N_2O_3$: C, 72.28; H, 4.85; N, 8.43. Found: C, 72.54; H, 4.89; N, 8.47.

7.4. General procedure for monohalogenation reaction

A solution of the intermediate **48** or **52** (1.1 mmol) in $CHCl_3$ was treated with 1 mmol of the halogen X_2 and the reaction mixture was stirred at 50 °C for 2 h. The solution was poured into water and extracted with dichloromethane. The organic layer was dried and concentrated. The residue was purified by flash chromatography on silica gel (hexane/ethyl acetate 1:1) to obtain the corresponding product.

7.4.1. 3-Benzyl-5-(bromoacetyl)benzamide (21). (0.07 g, 0.21 mmol, 68%). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 8.24 (t, J = 1.68 Hz, 0 H), 7.96 (t, J = 1.68 Hz, 1 H), 7.91 (t, J = 1.68 Hz, 1 H), 7.12–7.38 (m, 5 H), 6.04 (br s, 2 H), 4.43 (s, 2 H), 4.10 (s, 2 H). MS (ESI) *m/e* 332.2 [(M+H$^+$)]. HPLC t_R 13.8 min; purity 98% pkb-100 254 × 4.6 mm 5 μm, gradient elution of 30–60% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 min, λ = 216 nm. Anal. Calcd for $C_{16}H_{14}BrNO_2$: C, 57.85; H, 4.25; N, 4.22. Found: C, 57.71; H, 4.18; N, 4.17.

7.4.2. 3-(Bromoacetyl)-5-(naphth-2-ylmethyl)benzamide (22). (0.13 g, 0.34 mmol, 52%) ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.35 (t, J = 1.61 Hz, 1 H), 8.14 (br s, 1 H), 8.06–8.11 (m, 2 H), 7.78–7.90 (m, 4 H), 7.38–7.56 (m, 4 H), 4.96 (s, 2 H), 4.26 (s, 2 H). MS (ESI) *m/e* 382.1 [(M+H$^+$)]. HPLC t_R 12.4 min; purity 93% pkb-100 254 × 4.6 mm 5 μm, gradient elution of 35–65% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 min, λ = 225 nm. Anal. Calcd for $C_{20}H_{16}BrNO_2$: C, 62.84; H, 4.22; N, 3.66. Found: C, 62.71; H, 4.24; N, 3.53.

7.4.3. 3-Benzyl-5-(chloroacetyl)benzamide (13). (0.065 g, 0.22 mmol, 15%) ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.31 (s, 1 H), 8.12 (br s, 1 H), 8.05 (s, 1 H), 8.00 (s, 1 H), 7.51 (br s, 1 H), 7.14–7.38 (m, 5 H), 5.21 (s, 2 H), 4.08 (s, 2 H). MS (ESI) *m/e* 288.1 [(M+H$^+$)]. HPLC t_R 12.6 min; purity 96% synergy hydro-RP 250 × 4.6 mm 4 μm, gradient elution of 70–40% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 min, λ = 216 nm. Anal. Calcd for $C_{16}H_{14}ClNO_2$: C, 66.79; H, 4.90; N, 4.87. Found: C, 67.18; H, 5.11; N, 4.54.

7.4.4. 3-(Chloroacetyl)-5-(naphth-2-ylmethyl)benzamide (14). (0.02 g, 0.06 mmol, 45%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.31 (t, J = 1.53 Hz, 1 H), 8.12 (br s, 1 H), 8.09 (t, 1 H), 8.06 (t, J = 1.61 Hz, 1 H), 7.82–7.91 (m, 3 H), 7.80 (s, 1 H), 7.45–7.53 (m, 3 H), 7.42 (dd, J = 8.48, 1.75 Hz, 1 H), 5.21 (s, 2 H), 4.25 (s, 2 H). MS (ESI) *m/e* 338.2 [(M+H$^+$)]. HPLC t_R 15.5 min; purity 97% pkb-100 254 × 4.6 mm 5 μm, gradient elution of 30–60% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 minutes, λ = 225 nm. Anal. Calcd for $C_{20}H_{16}ClNO_2$: C, 71.11; H, 4.77; N, 4.15. Found: C, 69.95; H, 4.67; N, 3.97.

7.4.5. 5-(Bromoacetyl)-*N*-phenyslisophthalamide (23). (0.09 g, 0.25 mmol, 60%) ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 10.53 (s, 1 H), 8.71 (t, J = 1.57 Hz, 1 H), 8.65 (d, J = 1.74 Hz, 1H), 8.29 (br s, 1 H), 7.79 (d, J = 7.49 Hz, 2 H), 7.68 (br s, 1 H), 7.39 (t, J = 7.93 Hz, 2 H), 7.14 (t, J = 7.32 Hz, 1 H), 5.06 (s, 2H). MS (ESI) *m/e* 361.2 [(M+H$^+$)]. HPLC t_R 14.3 min; purity 82% pkb-100 254 × 4.6 mm 5 μm, gradient elution of 20–50% CH_3CN/CH_3COONH_4 10 mM for 30 min, λ = 216 nm. Anal. Calcd for $C_{16}H_{13}BrN_2O_3$: C, 53.21; H, 3.63; N, 7.76: Found: C, 53.49; H, 3.71; N, 7.62.

7.4.6. 5-(Bromoacetyl)-N-2-naphthylisophthalamide (24). (0.087 g, 0.21 mmol, 68%) ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 10.71 (s, 1 H), 8.83 (t, J = 1.65 Hz, 1 H), 8.79 (t, J = 1.65 Hz, 1 H), 8.64–8.72 (m, 2 H), 8.32 (br s, 1 H), 7.95–8.06 (m, 2 H), 7.90 (d, J = 8.05 Hz, 1 H), 7.52–7.65 (m, 4 H), 5.08 (s, 2 H). MS (ESI) *m/e* 411.1 [(M+H$^+$)]. HPLC t_R 12.4 min; purity 53% pkb-100 254 × 4.6 mm 5 μm, gradient elution of 25–55% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 min, λ = 218 nm. Anal. Calcd for $C_{20}H_{15}BrN_2O_3$: C, 58.41; H, 3.68; N, 6.81. Found: C, 58.50; H, 3.72; N, 6.69.

7.5. General procedure for dihalogenation reaction

A solution of the intermediate **48** or **52** (1 mmol) in $CHCl_3$ was treated with 2 mmol of the halogen X_2 and the reaction mixture was stirred at 50 °C for 2.5 h. The solution was poured into water and extracted with dichloromethane. The organic layer was dried and concentrated. The residue was purified by flash chromatography on silica gel (hexane/ethyl acetate 1:1) to obtain the corresponding product.

7.5.1. 3-Benzyl-5-(dibromoacetyl)benzamide (16). (0.06 g, 0.145 mmol, 65%) ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.38 (s, 1 H), 8.11 (s, 2 H), 8.07 (s, 1 H), 7.82 (s, 1 H), 7.54 (br s, 1 H), 7.12–7.39 (m, 5 H), 4.08 (s, 2 H). MS (ESI) *m/e* 441.9 [(M+H$^+$)]. HPLC t_R 14.2 min; purity 98% pkb-100 254X4.6 mm 5 μm, gradient elution of 35–65% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 min, λ = 258 nm. Anal. Calcd for $C_{16}H_{10}Br_2NO_2$: C, 46.75; H, 3.19; N, 3.41. Found: C, 46.93; H, 3.04; N, 3.41.

7.5.2. 3-(Dibromoacetyl)-5-(naphth-2-ylmethyl)benzamide (17). (0.04 g, 0.09 mmol, 15%) ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.38 (s, 1 H), 8.11 (s, 2 H), 8.07 (s, 1 H), 7.82 (s, 1 H), 7.78–7.90 (m, 3H), 7.54 (br s, 1 H), 7.38–7.56 (m, 4H), 4.08 (s, 2 H). MS (ESI) *m/e* 461.9 [(M+H$^+$)]. HPLC t_R 16.5 min; purity 94% pkb-100 254 × 4.6 mm 5 μm, gradient elution of 35–65% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 min, λ = 225 nm. Anal. Calcd for $C_{20}H_{15}Br_2NO_2$: C, 52.09; H, 3.28; N, 3.04. Found: C, 51.89; H, 3.28; N, 2.73.

7.5.3. 5-(Dibromoacetyl)-*N*-phenylisophthalamide (18). (0.003 g, 0.007 mmol, 65%) ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 10.53 (s, 1 H), 8.71 (t, J = 1.57 Hz, 1 H), 8.65 (d, J = 1.74 Hz, 1H), 8.29 (br s, 1 H), 7.79 (d, J = 7.49 Hz, 2 H), 7.68 (br s, 1 H), 7.39 (t, J = 7.93 Hz, 2 H), 7.14 (t, J = 7.32 Hz, 1 H), 7.6 (s, 1H). MS (ESI) *m/e* 441.1 [(M+H$^+$)]. HPLC t_R 11.3 min; purity 93% pkb-100 254 × 4.6 mm 5 μm, gradi-

ent elution of 20–65% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 min, $\lambda = 208$ nm. Anal. Calcd for $C_{16}H_{12}Br_2N_2O_3$: C, 43.67; H, 2.75; N, 6.37. Found: 43.75; H, 2.80; N, 6.41.

7.5.4. 5-(Dibromoacetyl)-*N*-2-naphthyliphthalamide (19). (0.04 g, 0.08 mmol, 45%) ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 10.70 (s, 1 H), 8.82–8.87 (m, 3 H), 8.72–8.76 (m, 1 H), 8.46 (br s, 0 H), 7.97–8.08 (m, 3 H), 7.86–7.94 (m, 2 H), 7.51–7.63 (m, 4 H). MS (ESI) *m/e* 489.2 [(M+H$^+$)]. Anal. Calcd for $C_{20}H_{14}Br_2N_2O_3$: C, 49.01; H, 2.88; N, 5.72. Found: C, 49.32; H, 3.01; N, 5.63.

7.5.5. Methyl 3-amino-5-carbamoylbenzoate (54). 3-(methoxycarbonyl)-5-nitrobenzoic acid (11.2 g, 49.7 mmol) and toluene (90 mL) were placed in a three bottom flask equipped with a condenser and a magnetic stirrer. The resulting mixture was vigorously stirred at room temperature and thionyl chloride (12.7 mL, 175 mmol) was slowly added. The solution was refluxed for 3 h. After being cooled at room temperature the solvent was evaporated in vacuo. The crude residue was used for the next reaction without further purification.

The chloride was placed in a three bottom flask equipped with a condenser and a magnetic stirrer, at 0 °C and NH_3 32% (300 mL) and was added in 30′. The solution was stirred at rt for 2 days. The precipitate was filtered and washed with NaOH 1 N and dissolved in hot acetonitrile. The mixture was filtered and the solvent was evaporated in vacuo obtaining the amide.

To a solution of methyl 3-carbamoyl-5-nitrobenzoate (6 g, 26.8 mmol) in abs EtOH (90 mL) $SnCl_2H_2O$ (30.3 g, 134 mmol) was added. The solution was stirred at 70 °C for 1 h and poured into a $NaHCO_3$ ice bath. The precipitate was filtered off and the alkaline solution was extracted with ethyl acetate. The organic layer was dried and concentrated obtaining **54** (2.6 g, 13.4 mmol, 50%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 7.91 (br s, 1 H), 7.58 (t, $J = 1.53$ Hz, 1 H), 7.31 (dd, $J = 2.34$, 1.61 Hz, 1 H), 7.27 (dd, $J = 2.34$, 1.61 Hz, 1 H), 7.22 (br s, 1 H), 5.52 (s, 2 H), 3.83 (s, 3 H). GC–MS (70 eV; rel Int): 194 (100) [M$^+$], 178 (48), 91 (38).

7.5.6. Methyl 3-(benzylamino)-5-carbamoylbenzoate (25), methyl 3-carbamoyl-5-(dibenzylamino)benzoate (26). A solution of benzyl chloride (0.852 mL, 7.4 mmol) in toluene (10 mL) was added dropwise to a solution of **54** (1.3 g, 6.69 mmol) and Et_3N (1.03 mL, 7.4 mmol) in toluene (50 mL) and the reaction mixture was refluxed overnight. After being cooled at room temperature the precipitated white solid was filtered and it showed to be a mixture of compounds **25** and **26**. They were separated by flash chromatography on silica gel (chloroform/methanol 95:5) to obtain **25** (0.4 g, 1.4 mmol, 21%) and **26** (0.2 g, 0.5 mmol, 7%). **25** ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 7.95 (br s, 1H), 7.62 (t, $J = 1.46$ Hz, 1H), 7.18–7.40 (m, 8H), 6.73 (t, $J = 6.07$ Hz, 1H), 4.35 (d, $J = 5.99$ Hz, 2H), 3.82 (s, 3H). MS (ESI) *m/e* 285.2 [(M+H$^+$)]. HPLC t_R 13.5 min; purity 96% pkb-100 254×4.6 mm 5 μm, gradient elution of 20–50% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 min, $\lambda = 277$ nm. Anal. Calcd for $C_{16}H_{16}N_2O_3$: C, 67.59; H, 5.67; N, 9.85. Found: C, 67.42; H, 5.61; N, 9.78. **26** ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 8.01 (br s, 1H), 7.70 (s, 1H), 7.47 (dd, $J = 2.56$, 1.39 Hz, 1H9, 7.18–7.41 (m, 12H), 4.78 (s, 4H), 3.78 (s, 3H). MS (ESI) *m/e* 375.3 [(M+H$^+$)]. HPLC t_R 16.7 min; purity 95% pkb-100 254×4.6 mm 5 μm, gradient elution of 30–60% CH_3CN (0.01% TFA)/H_2O (0.01% TFA) for 30 min, $\lambda = 285$ nm. Anal. Calcd for $C_{23}H_{22}N_2O_3$: C, 73.78; H, 5.92; N, 7.48. Found: C, 73.44; H, 6.03; N, 7.34.

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