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Improved Flavodoxin Inhibitors with Potential Therapeutic Effects against *Helicobacter pylori* infection

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ABSTRACT: *Helicobacter pylori* (*Hp*) infection affects one half of the human population and produces a variety of diseases from peptic ulcer to cancer. Current eradication therapies achieve modest success rates (around 70 %), resistances to the antibiotics of choice are on the rise, and vaccination has not proved successful yet. Using an essential *Hp* protein, flavodoxin, as target, we identified three low-MW flavodoxin inhibitors with bactericidal anti-*Hp* properties. To improve their therapeutic indexes we have now identified and tested 123 related compounds. We have first tested similar compounds available. Then, we have designed, synthesized and tested novel variants for affinity to flavodoxin, MIC for *Hp*, cytotoxicity, and bactericidal effect. Some are novel bactericidal inhibitors with therapeutic indexes of 9, 38 and 12, significantly higher than those of their corresponding leads. Developing novel *Hp*-specific antibiotics will help fighting *Hp* resistances and may have the advantage of not generally perturbing the bacterial flora.

Introduction

Helicobacter pylori (*Hp*) is a gram negative bacteria that establishes life-long infections in the gastric mucosa of humans.[1, 2] In many cases, without specific antimicrobial intervention, *Hp* infected individuals will develop type B gastritis, chronic peptic ulcers and, more rarely, gastric neoplasias. As stated in the Maastricht IV/Florence Consensus Report:[3] "*H. pylori* is the most successful human pathogen, infecting an estimated 50% of the global population." The prevalence of the infection varies from place to place, average prevalence in Europe being around 30% and much higher in developing countries. Individuals infected with *Hp* have a 10 to 20% lifetime risk of developing peptic ulcers and a 1 to 2% risk of developing stomach cancer. Eradication of *Hp* infection is recommended in peptic ulcer disease, low grade gastric mucosa associated lymphoid tissue (MALT) lymphoma, atrophic gastritis, first degree relatives of patients with gastric cancer, unexplained iron deficiency anemia, and chronic idiopathic thrombocytopenic purpura. Besides, it has been pointed out that *Hp* eradication may prevent peptic ulcer in naïve users of non-steroidal anti-inflammatory drugs (NSAIDs).[4]

The standard therapy used worldwide to eradicate *Hp*, known as the triple treatment,[5] consists of a combination of one proton pump inhibitor (PPI) with two wide spectrum antibiotics: clarithromycin and a choice of amoxicillin or metronidazole. Unfortunately, this combination has lost efficacy and, at present, it allows the cure of a maximum of 70 % of the patients. The reason for this loss of efficacy appears to be the increase in clarithromycin and/or metronidazole resistance in *Hp*. Besides, in developing countries eradication therapies face the additional problems of high cost, high prevalence of strains resistant to available antibiotics,[6, 7] and/or high reinfection rates due to poor socioeconomic and sanitation policies. As indicated: "A wider range of effective treatments is urgently required."[3] In contrast with needs, no new drugs have been developed for this indication in recent years and, significantly enough, there is not a single specific *Hp* antimicrobial.

Novel molecules, synthetic or natural, showing anti-*Hp* activity have been described elsewhere (see reference 8 for a recent review). Often, such novel compounds are not identified as active against specific *Hp* validated targets, which complicates their further development and, so far, they have not resulted in novel antibiotics. Vaccination is a clear alternative to combat *Hp* infection but, although there have been many initiatives to obtain *Hp* vaccines, no one has succeeded yet. A recent review on this approach[9] states: "...vaccine development against *H. pylori* remains a focus of research. Progress is made but is incremental. There is need for a still better understanding of the protective mechanism and for improving efficacy". Given that the existing anti-*Hp* products (wide spectrum antibiotics typically used in combinations) currently achieve only a 70 % success in eradication, novel, ideally *Hp*-specific small molecules are strongly needed to face the challenge of growing resistances. On the other hand, development of anti-

biotics specific to a given bacteria may be advantageous in that it may reduce the characteristic side-effect of antibiotic therapy consisting in disruption of the natural human bacterial flora.[10]

Several groups have attempted to develop narrow-spectrum antimicrobials against *Hp* and have indeed identified selective targets in this organism.[11] One of them is the protein flavodoxin (*Hp*-Fld), whose function is essential for *Hp* viability.[12, 13] Flavodoxins are electron carriers involved in a wide range of bacterial reactions, and they contain a redox active flavin mononucleotide molecule (FMN) noncovalently bound.[14] *Hp*-Fld is involved in the oxidative decarboxylation of pyruvate leading to synthesis of NADPH. In this reaction, *Hp*-Fld shuttles electrons from the pyruvate:ferredoxin oxidoreductase complex (PFOR)[15] to flavodoxin:quinone reductase (FqrB).[16] Structurally, *Hp*-Fld differs from other flavodoxins in that it forms a distinct pocket near the cofactor binding site (**Figure 1**) where an alanine residue replaces the structurally equivalent bulky residue typically present in other flavodoxins.[13] We proposed[12] that the pocket could allow a selective binding of inhibitors that could interfere with flavodoxin function, and accordingly screened a 10.000-compound collection using an *in vitro* protein thermostability assay.[17] Thus, we identified 29 compounds that specifically bind to *Hp*-Fld and could in principle inhibit its function. The 29 binders were tested in a flavodoxin functional assay performed in anaerobiosis in presence of the PFOR and FqrB enzymes, which allowed to identify four flavodoxin inhibitors. Those four compounds proved to inhibit the growth of *Hp* cultures and three of them showed bactericidal effects.[17]

We describe here the identification/design and testing of 123 compounds related to those three bactericidal compounds, which has led to the discovery of novel bactericidal compounds with significantly greater therapeutic indexes.

Inhibitor candidates. The compounds tested include three previously reported flavodoxin inhibitors,[17] compounds **I**, **II** and **IV**, plus 123 related compounds: 30 analogues of **I**, 62 analogues of **II** and 31 analogues of **IV**. Analogues of **I** included 22 compounds identified by similarity searches in the *Maybridge* catalogue (http://www.maybridge.com) (Supporting Information: **Table S1**, compounds **1-4, 8-25**); 2 compounds identified by similarity searches in *SciFinder* database (http://scifinder.cas.com)(**Table S1**, compounds **5**, **26**); and 6 compounds purposely designed which were synthesized by *Maybridge* (**Table S1**, compounds **6-7, 27-30**). Analogues of **II** included 44 compounds identified by similarity searches in the *Maybridge* catalogue (**Table S1**, compounds **31-32, 46-87**); 10 compounds indentified by similarity searches in *Scifinder* database (**Table S1**, compounds **33-39**, **88-90**); and 8 compounds purposely designed, which were synthesized by *Maybridge* (**Table S1**, compounds **40-45**, **91, 92**). Analogues of **IV** included 19 compounds identified by similarity searches in the *Maybridge* catalogue (**Table S1**, compounds **93-110, 122**); 5 compounds indentified by similarity searches in *Scifinder* (**Table S1**, compounds **111-115**); and 7 compounds purposely designed, which were synthesized by *Maybridge* (**Table S1**, compounds **116-121, 123**). The 17 compounds identified in *SciFinder* data base were either purchased from commercial sources or kindly supplied by a variety of laboratories (see suppliers in **Table S1**).

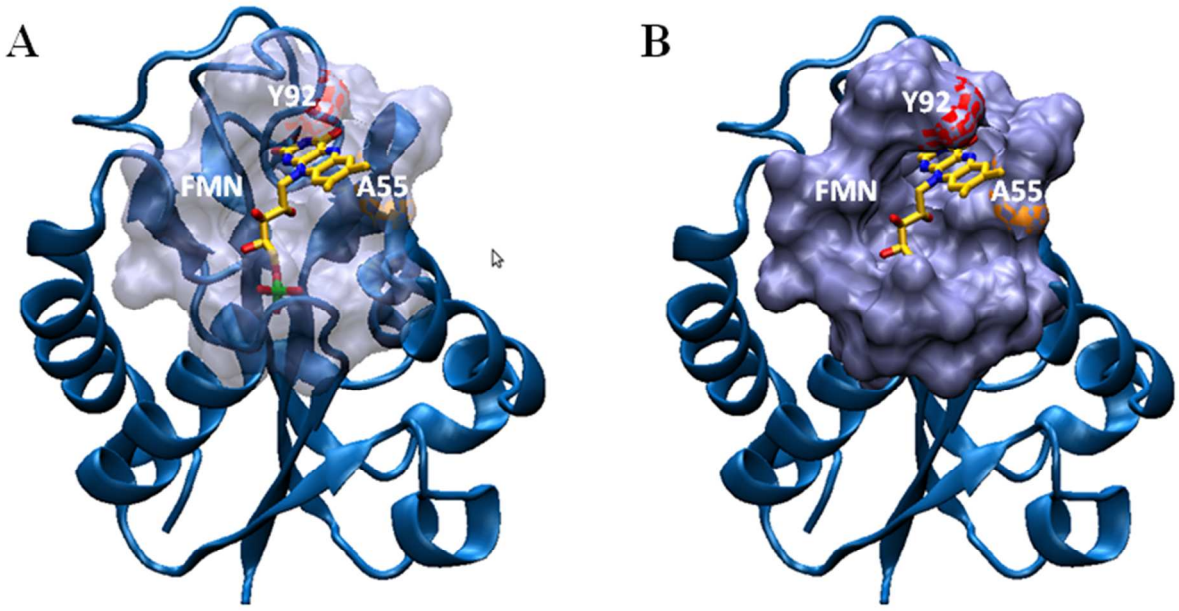
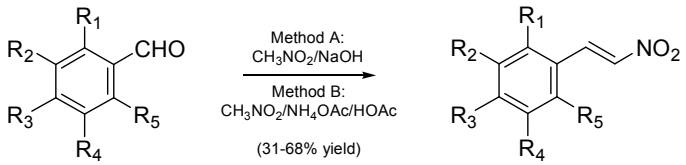


Figure 1. *Helicobacter pylori* flavodoxin X-ray structure. A) Ribbon drawing showing the structure of *Hp*-Fld and a transparent molecular surface of the cofactor binding site (the FMN cofactor in sticks). B) Molecular surface of the cofactor binding site showing the FMN-interacting Y92 residue and A55. *Hp*-Fld A55 appears at a position where bulkier residues are typically located in other flavodoxins, thereby helping to create a pocket near the active site where it is believed the inhibitors to bind.[17]

Synthesis. Briefly, the custom-made synthesis performed by *Maybridge Chemical Company, Ltd*. leading to the new analogues was as follows.

Schemes 1 and 2 show the synthetic routes to new analogues of **II**. Nitrostyrene derivatives **40-45** and **91** were accessible via condensation of nitromethane with appropriate aldehydes (see **Table S1** for identities of R_1-R_5) under either base catalyzed (method A, for **40-42** and **44**)[18] or acid catalyzed conditions (method B, for **43**, **45** and **91**).[19]

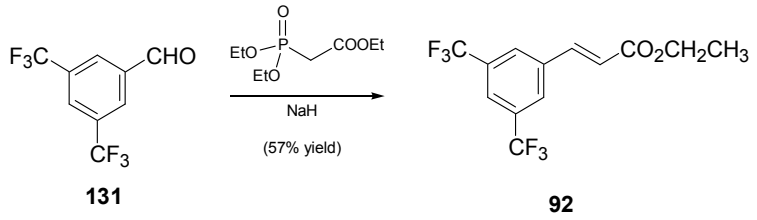
Scheme 1



124-130 **40-45, 91**

Compound **92** could be readily prepared via Horner-Wadsworth-Emmons olefination[20] of the appropriate aldehyde **131**.

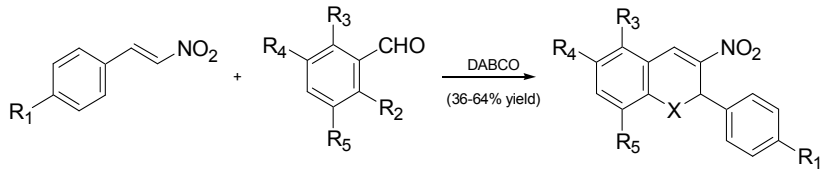
Scheme 2



131 **92**

Analogues of **I** were prepared according to Schemes 3 and 4. Some of the already synthesized nitrostyrene derivatives (**43-45**; see **Table S1** for identities of R_1) were further used to obtain compounds **6** and **28-30**, via Baylis–Hillman reaction[21] with the appropriate aldehydes (**132-134;** see **Table S1** for identities of R_2-R_5 and X).

Scheme 3

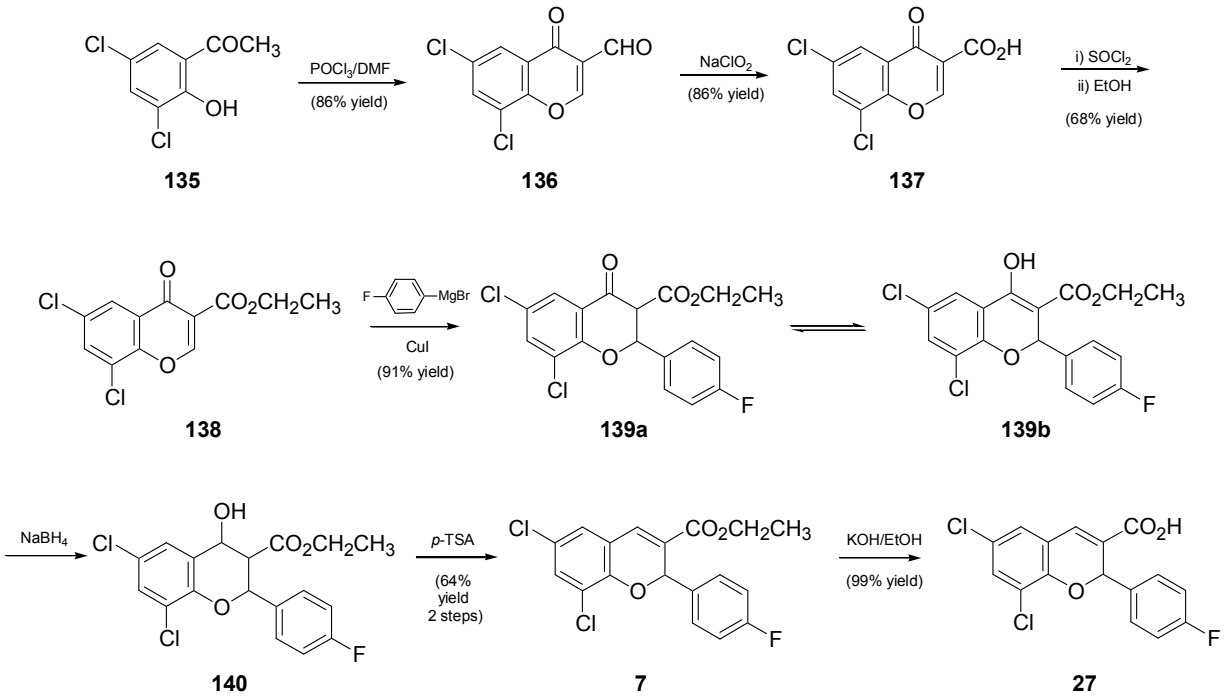


43-45 **132-134** **6, 28-30**

The synthesis of compounds **7** and **27** is described in Scheme 4. First step consists of a Vilsmeier-Haack reaction[22] on **135**, followed by oxidation[23] of the resulting aldehyde **136**. Further conversion of the carboxylic acid **137** to the ethyl ester **138** was achieved in a one pot reaction.[24] This was followed by Grignard reaction[25] of **138** with 1-bromo-4-fluorobenzene, which gave the desired compound **139** as a mixture of keto-enol tautomers (**139a** and **139b**). Subsequent reduction of the mixture with sodium borohydride[26] afforded the alcohol **140** and also some of the desired ester **7**. This crude mixture of alcohol and ester was dehydrated with *p*-toluensulphonic acid,[27] leading to compound **7,** which was quantitatively hydrolyzed under basic conditions[28] to obtain compound **27**.

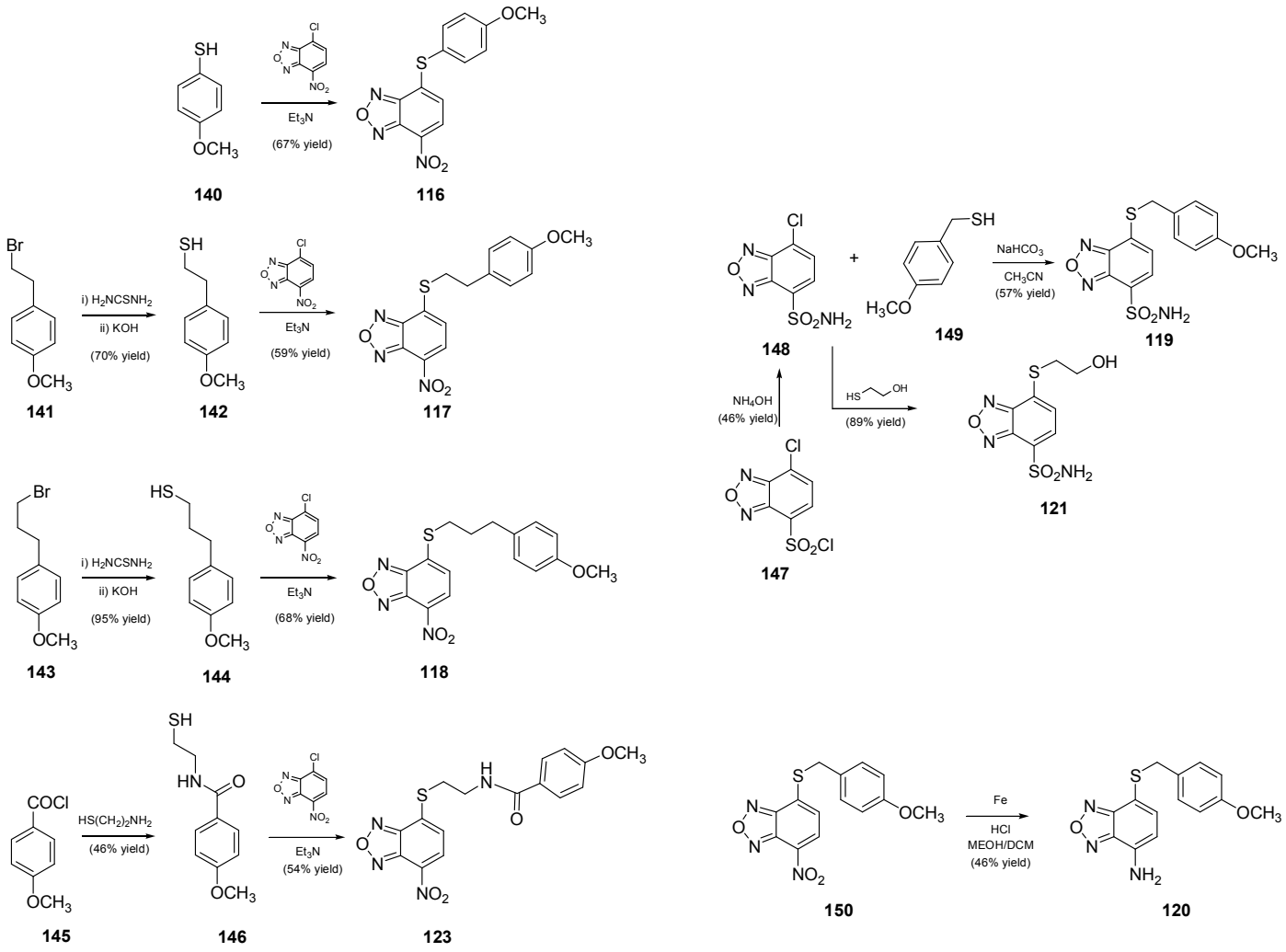
The synthesis of analogues of **IV** is described in Scheme 5. Compounds **116-119, 121** and **123** could be obtained applying the same strategy, consisting of a reaction of the appropriate thiols with the appropriate chlorobenzoxadiazoles under basic conditions.[29] The thiols required for the synthesis of **117** and **118** were **142** and **144** respectively, which were prepared from **141** and **143** by reaction with thiourea and subsequent hydrolysis[30]. Thiol **146**, that was used for the synthesis of **123**, was obtained from **145** by reaction with 2-aminoethanothiol.[31] Also, one of the intermediates in the synthesis of **119** was further used for the preparation of **121**.[32] Finally, **120** was obtained by reduction[33] of the convenient commercially available nitrobenzoxadiazole **150**.

Scheme 4



The synthesis of analogues of **IV** is described in Scheme 5. Compounds **116-119, 121** and **123** could be obtained applying the same strategy, consisting of a reaction of the appropriate thiols with the appropriate chlorobenzoxadiazoles under basic conditions.[29] The thiols required for the synthesis of **117** and **118** were **142** and **144** respectively, which were prepared from **141** and **143** by reaction with thiourea and subsequent hydrolysis.[30] Thiol **146**, that was used for the synthesis of **123**, was obtained from **145** by reaction with 2-aminoethanothiol.[31] Also, one of the intermediates in the synthesis of **119** was further used for the preparation of **121**.[32] Finally, **120** was obtained by reduction[33] of the convenient commercially available nitrobenzoxadiazole **150**.

Scheme 5



RESULTS

Prescreening of available analogues for binding to Hp flavodoxin, and affinities of the complexes. The derivatives of **I**, **II** and **IV** which were available from *Maybridge*, and those identified in *SciFinder* and commercially available or gifted to us by academic laboratories were initially tested for binding to *Hp*-Fld using a fast thermostabilization assay implemented in the previously reported HTS.[17] The compounds that increased the *Hp*-Fld *Tm* by at least 2 standard deviations of the mean *Tm* obtained for the control samples (with no compound added) were selected for further testing. They include analogues of **I (1-5)**, analogues of **II (31-38)**, and analogues of **IV (93, 102, 105-108** and **110)**. The affinity of these compounds for *Hp*-Fld was then determined by ITC. The analogues of **I** selected from their thermostabilization of flavodoxin were shown by ITC to form 1:1 complexes with the protein with K_d*s* from 0.4 to 1.9 μM, in the same range that the K_d of the complex formed between flavodoxin and **I**: 1.0 μM (**Table 1**). The selected analogues of **II** formed 1:1 complexes with flavodoxin with K_d*s* covering a wider range of values (from 0.7 to 19 μM)

around the value measured for the complex between flavodoxin and **II**: 3.5 μM. The selected analogues of **IV** also established 1:1 complexes with flavodoxin with K_ds from 0.2 to 9.6 μM, around the K_d value for the complex between flavodoxin and **IV**: 1.7 μM.

MICs and bactericidal properties of derivatives. The selected *Hp*-Fld binders were tested as inhibitors of *Hp* growth by determining their MICs. Out of the 20 binders identified, 19 were effective inhibiting *Hp* growth, with MICs ranging from 1.2 to 7.5 μM for analogues of **I**, from 2.4 to 8 μM for analogues of **II**, and from 4.8 to 38 μM for analogues of **IV** (**Table 1**). Kill curves determined for some of the *Hp* inhibitors showing the highest therapeutic indexes (computed using the MICs and the toxicity data (MCC) described below) indicated that at least two analogues of **I** (**4** and **5**), one analogue of **II (31)**, and two analogues of **IV** (**105** and **108**) displayed bactericidal properties.

Toxicity of derivatives and therapeutic indexes. The toxicity for HeLa cells of the 19 binders that inhibit *Hp* growth was determined using the XTT assay. The corresponding MCCs are reported in **Table 1**.

They range from 0.01 μM for the most toxic compound to 100 μM for several less toxic compounds. With the exception of three derivatives of **II**, all the MCCs were ≥1 μM. The TIs corresponding to derivatives of **I** ranged from 0.4 to 9.3, those for derivatives of **II**, from 0.001 to 8.4, and those for derivatives of **IV**, from 0.2 to 11.

Design and testing of novel analogues. The thermostabilization (not shown) and the binding data obtained using the available analogues of **I, II** and **IV** was qualitatively analyzed in order to design novel variants. For compound **I**, substitution of chlorine at position 6 in the chromene moiety appeared preferred to substitution of chlorine at position 8, while in the fluorophenyl moiety, fluorosubstitution was preferred to introduction of substituents at other positions (not shown). Variants were designed to test the importance of the nitro group for binding and activity (**27** and **7**), to test different halogen replacements for the fluorine (**29** and **6**), to test replacement of the chromene oxygen atom by nitrogen (**28**), and to introduce substituents at other positions in chromene ring (**30**) (Tables 2 and S1). Replacement of the nitro group by a carboxylic acid (**27**) lowered the affinity for flavodoxin and formation of complex was not observed by ITC (not shown). Esterification (**7**) restored binding with K_d similar to that of **I**. The toxicity of **7** was slightly lower than that of **I** but, unfortunately, the MIC was greatly increased, leading to a lower TI compared to **I**. Introduction of a chlorine at position **5 (30)** led to loss of binding (not shown).

Replacement of the fluorine by either bromine or iodine (**29, 6**), or replacement of the oxygen by nitrogen (**28**) lowered the affinity by one order of magnitude (K_d for **6:** 25 μM (**Table 1**), K_d for **28** and **29**, 33 and 25 μM respectively). The iodine containing derivative (**6**) displayed, nevertheless, similar MIC, toxicity and TI than **I**.

For compound **II**, the preliminary thermostabilization and the affinity data suggested that the trifluoromethyl groups could be removed or replaced, and that alkylation of the vinyl moiety was possible. Replacement of the nitro group by a carboxylic acid (**39**) reduced the affinity, but complex formation with flavodoxin was still observed. Although **39** was less toxic than **II**, it was also less

effective (higher MIC) and the TI was lower. Esterification (**92**) (see Table S1) greatly reduced the affinity, and no complex formation was observed by ITC (not shown). Removal of one CF_3 group and insertion of a chlorine at *para* position to the nitrovinyl substituent (**40**) hardly changed affinity, inhibition or toxicity and the compound exhibited a TI similar to that of **II**.

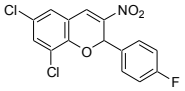
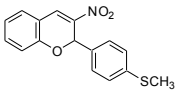
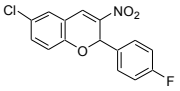
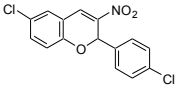
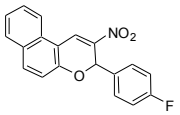
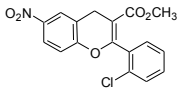
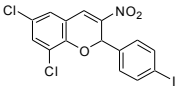
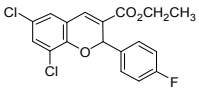
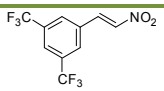
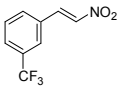
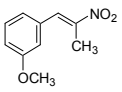
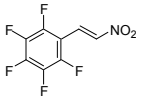
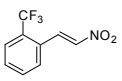
Replacement of both CF_3 groups by one chlorine and one bromine (**91**) (see Table S1) left the affinity and toxicity almost unchanged (Kd of 10 μM and MCC of 2.7 μM). In compound **41** one of the CF_3 groups was removed and fluorine was introduced in position para to the remaining CF_3. This compound displayed similar affinity, lower toxicity and lower MIC, thus displaying a higher TI (~15) than **II**. Compound **42** contained no CF_3 groups but two fluorines (both ortho to the nitrovinyl substituent) plus one chlorine (para to the nitrovinyl substituent). This compound exhibited a slightly lower affinity but was more potent (lower MIC) and one order of magnitude less toxic than **II**. Its TI (~38) was thus higher. Finally, three compounds were tested that contained no CF_3 groups but just one halogen (in para position): either fluorine, or bromine or iodine (**45, 43** and **44**, respectively). Only the fluorine containing compound displayed a clearly lower affinity than **II**. The three compounds were as inhibitory or more than **II** and they exhibited lower toxicity. Accordingly their TIs were higher than those of **II**.

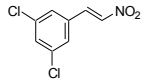
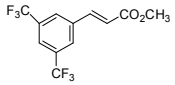
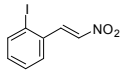
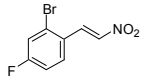
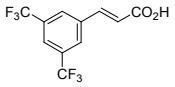
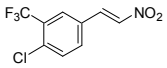
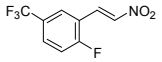
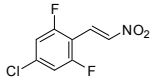
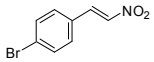
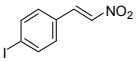
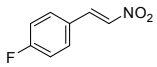
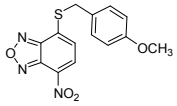
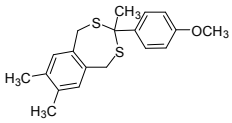
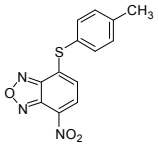
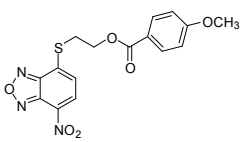
The preliminary analysis of derivatives of **IV** indicated that the p-methoxyphenyl moiety could be modified and that the spacing between this moiety and the sulphur atom could be changed. The relevance of the spacing was tested by directly connecting the *p*-methoxyphenyl group to the sulphur atom (**116**), and by replacing the methylene group in **IV** by either an ethylene (**117**) or a propylene group (**118**). Only compound **117** (see Table S1) showed no affinity for flavodoxin (not shown), **116** displaying a similar K_d and **118** being significantly less affine but nevertheless binding to the protein. The spacing was also modified by introducing an amide linkage (compound **123**, see Table S1) between the two aromatic systems in **IV**, but this compound did not apparently bind to flavodoxin (not shown). Of these four derivatives, **116** exhibited the highest TI (=1.5), which although admittedly small, is higher than that of **IV** (~0.2).

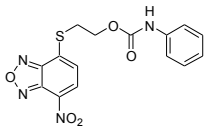
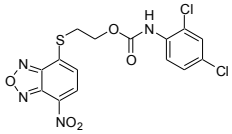
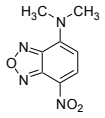
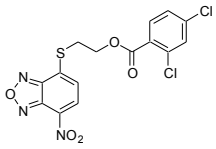
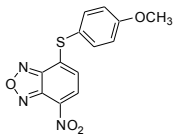
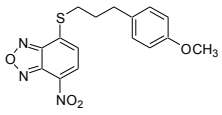
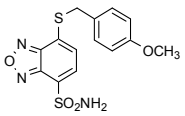
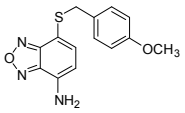
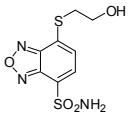
Replacement of the nitro group by a sulphonamide group (**119**) improved the affinity, lowered the toxicity and slightly improved the inhibition, leading to an improved TI of 0.6. Replacement of the nitro group by an amine group (**120**) decreased the affinity, did not modify the inhibition and markedly reduced the toxicity, which combined to a much higher TI of 12. Two short versions of **IV** were additionally tested. In one of them (**121**), the nitro group was replaced by a sulfonamide group and the *p*-methoxyphenyl moiety was replaced by a hydroxymethyl group. This compound can still bind flavodoxin (with lower affinity) and displays a low toxicity, but it cannot inhibit the grown of *Hp* in culture. In the other one, only the benzoxadiazole ring remains, with the oxygen changed by sulphur and one bromine as the only substituent (**122**). Its properties are very similar to those of the other shortened compound and it does not inhibit *Hp* cells growth.

The bactericidal properties of some of the novel compounds exhibiting high TI have been tested. Compounds **42-45**, **116** and **120** are bactericidal.

Table 1. Observed values for tested BRs in selected compounds.

Ca[p]	Structure	K_d	MIC (μM)	MCC (μM)	TI[b]
I		1	1.2	6	5.0
1[c]		0.43	2.4	1	0.4
2[c]		1.5	2.4	6	2.5
3[c]		1.7	2.4	6	2.5
4[c]		0.78	1.2	10	8.3
5[c]		1.9	7.5	70	9.3
6[c]		25	2	10	5.0
7[c]		1.8	>150	12	<0.08
II		3.5	2.4	3.5	1.5
31[d]		7.9	4.75	40	8.4
32[d]		5.5	2.4	0.70	0.3
33[d]		10	8	0.01	0.001
34[d]		8.8	2.4	0.1	0.04

35[d]		0.67	2.4	2	0.8
36[d]		3.7	>150	100	<0.7
37[d]		16	4.8	1	0.2
38[d]		19	4.8	10	2.1
39[d]		11.0	75	100	1.3
40[d]		2.8	1.06	5	4.7
41[d]		4.4	0.53	8	15.1
42[d]		7.4	0.53	20	37.7
43[d]		4.1	0.53	8	15.1
44[d]		6.6	1.06	10	9.4
45[d]		40	1.06	20	18.9
IV		1.7	9.5	1.7	0.2
93[e]		9.6	9.5	100	10.5
102[e]		0.97	4.8	1.3	0.3
105[e]		0.22	9.5	8	0.8

106[e]		3.8	9.5	2.2	0.2
107[e]		1.9	9.5	1.7	0.2
108[e]		0.8	38	100	2.6
110[e]		2.8	19	4	0.2
116[e]		2.6	2	3	1.5
118[e]		18	-	1	-
119[e]		0.48	19	12	0.6
120[e]		7.4	8.5	100	11.8
121[e]		21.8	>150	100	<0.7
122[e]		4.8	>150	100	<0.7

[a] Number of compound given in **Table S1**.

[b] Values calculated as *MCC/MIC*.

[c,d,e] Derivatives of inhibitor **I**, **II** and **IV** respectively.

DISCUSSION

An initial test of the binding of existing derivatives of inhibitors **I, II** and **IV** to flavodoxin was performed using a HTS method.[17] The test allowed us to identify 5 (**1-5**), 8 (**31-38**) and 7 (**93, 102, 105-108** and **110**) novel binders, structurally related to, respectively, compound **I, II** and **IV** (**Table 1**).

In each of the three groups, there were compounds binding to flavodoxin with higher affinity than the original inhibitors. Most of these binders (19 out of 20) were effective inhibiting *Hp* growth and in each of the three groups there were bactericidal compounds. Interestingly, some of these inhibitors exhibited a significantly lower toxicity towards HeLa cells, which led to an increase in their TI. Based on the data gathered for these compounds, in some cases suggesting which parts of the molecules could be modified without compromising affinity for the flavodoxin target, we designed novel variants of inhibitors **I, II**, and **IV**, aiming at exploring their chemical space, while trying to introduce single modifications whenever possible and taking into account synthetic issues.

Analysis of activity data from the novel derivatives of **I** indicated that its nitro group could not be replaced by either a carboxylic acid or the corresponding ethyl ester without severely compromising either binding or inhibitory efficiency.

Similarly, for compound **II** such replacements of the nitro group reduced both the inhibitory activity and the cytotoxicity. Interestingly, the simultaneous removal of the two CF_3 groups with introduction of halogens in *para* markedly reduced the toxicity, greatly increasing the TI.

On the other hand, inhibitor **IV** contains two moieties (*benzoxadiazole* and *p-methoxyphenyl*) joined by a thioether link. The influence of link length was tested, and it seems that shorter links (with no or only one methylene) are better than longer ones. On the other hand, the nitro group could be replaced by a sulphonamide group with some improvement of the TI. More importantly, replacement of the nitro group by an amine markedly reduced the toxicity and therefore improved the TI. As for the *p*-methoxyphenyl moiety, two attempts to essentially remove it led to smaller molecules that could bind flavodoxin and displayed reduced toxicity, but lacked inhibitory activity. It should be mentioned that both of these variants lacked the nitro group.

The variants of **I**, **II** and **IV** exhibiting the highest TI within their groups were tested for bactericidal activity and they did kill *Hp* cells. They thus constitute improved versions of inhibitors **I, II** and **IV** with significantly expanded therapeutic windows.

CONCLUSIONS

Three known bactericidal compounds potentially useful to fight *Hp* infection have been improved using a two-round testing of derivatives. Initially, existing variants have been evaluated and, based on the results, novel variants have been designed and tested for affinity, toxicity, and inhibition of *Hp* growth. Overall, the therapeutic index of compound **I** has been increased from 5 to 9, that of **II** from 1.5 to 38, and that of **IV**, from 0.2 to 12. The derivatives of each compound exhibiting the highest TI are bactericidal and will be subjected to efficacy testing in an animal model to evaluate their feasibility as leads for novel specific antibiotics for the treatment of *Helicobacter pylori* associated diseases.

EXPERIMENTAL SECTION

Reagents and chemicals. All reagents and chemicals were obtained from commercial suppliers, and were used without any further purification. ^{1}H NMR and ^{19}F NMR spectra were acquired at room temperature at 400 and 376 MHz respectively, using a 5-mm probe. The chemical shifts (δ) are reported in parts per million from tetramethylsilane with the solvent resonance as the internal standard. Coupling constants (*J*) are quoted in hertzs. The splitting patterns are reported as: s (singlet), d (doublet), t (triplet), q (quartet), dd (doublet of doublets), m (multiplet), bs (broad singlet). Purity of compounds (made to order or kindly supplied by other laboratories) was determined to be ≥ 95% by HPLC chromatography, using a Waters HPLC system equipped with a 600-E pump, a 2996 PDA detector and a 2707 autosampler. The LC system was fitted with a C18 reversed-phase column (VYDAC 238TP C18 5μm, 4.6 mm x 250 mm) and operated, unless otherwise stated, using a linear gradient of buffer B (100% in 40 min) from 100% buffer A (buffer A: 0.1% TFA in H_2O, buffer B: 0.085% TFA in CH_3CN/H_2O 95:5 v/v) at a flow rate of 1mL/min.

***Hp* flavodoxin.** The flavodoxin from *H. pylori* was expressed in *E. coli* and purified as reported.[12] Protein concentration was determined from the absorbance of the FMN cofactor at 452 nm using an extinction coefficient of 10650 $M^{-1}cm^{-1}$.[12]

Preliminary testing for binding to *Hp* flavodoxin. For some of the inhibitor candidates, a preliminary flavodoxin binding test was performed in order to early discard compounds not binding to the target protein. This screening was based on comparing the temperature of mid-denaturation (T_m) of flavodoxin with that of flavodoxin in the presence of compound.[17] To that end, the intrinsic fluorescence of flavodoxin in the visible was monitored with a *FluoDia T70* spectrofluorimeter (excitation at 445 nm and emission at 525 nm) as a function of temperature. The flavodoxin FMN cofactor, strongly quenched by the apoprotein, is released as flavodoxin unfolds giving rise to a large increase in fluorescence emission.[34] The thermal unfolding curves were analyzed using software developed by us, and the apparent T_m*s* were calculated as reported.[17] Only the compounds that increased the T_m of the protein by at least 2 standard deviations of the mean T_m of protein controls (with no compound added) were further tested.

Determination of the affinity of complexes between *Hp*-Fld and candidate inhibitors. The dissociation constants of the complexes were determined at 25°C by Isothermal Titration Calorimetry (ITC**)** using a VP-ITC titration calorimeter (*MicroCal, GE Healthcare*). Degassed 20 μM flavodoxin solutions were titrated with concentrated inhibitor solutions (around 500 μM) dissolved in the same buffer (50 mM EPPS, pH 9.0 and 5% DMSO) and the heats associated with each injection were fitted assuming a 1:1 stoichiometry for the complex formed.

Minimal Inhibitory Concentrations: *MICs.* *Hp* strains 26695 and *Hp*1061 were grown as described.[16] For microdilution MIC determinations, 96 well round-bottom microtiter dishes were used. Bacteria growth and subsequent dilution to OD_{660}nm of 0.01

prior to mixing with appropriate concentrations of test compound were performed as described.[17] The low percentage of DMSO present in the assay (< 3% v/v) was not deleterious for the cells. Dishes were shaken under microaerobic conditions for 28 h. The lowest compound concentration completely inhibiting *Hp* growth was recorded as the MIC of the compound.

Bactericidal assays. The bactericidal activity of compounds towards *Hp* cells (strains 26695 and *Hp*1061) growing in *Brucella*-based medium supplemented with 7.5% fetal bovine serum was determined as previously described.[16] Bacteria were diluted to OD_{660nm}=0.1 in 3 mL of BHI broth supplemented with 2% newborn calf serum, and the appropriate small volume of test compound was added to a final test concentration of 2×MIC. Aliquots of the culture were removed at different time points, centrifuged, resuspended in BHI broth and plated on *Brucella* agar supplemented with 7.5% serum for determination of viable counts. Bactericidal activity was calculated following comparison with the DMSO control.

Minimal Cytotoxic Concentrations (*MCCs*). The toxicity of the compounds towards HeLa cells was determined by the XTT method using the *Cell Proliferation Kit II* (Roche), which detects dehydrogenase activity by reduction of a tetrazolium salt. All experiments were performed twice in triplicate. HeLa cells were cultured in complete medium (500 mL of Dulbecco's Modified Eagle Medium: P04-03591 from *Ibian Technologies;* plus 50 mL of bovine fetal serum; plus 5,5 mL of antibiotic containing 550000 units of penicillin and 550000 µg of streptomycin) with phenol red using 96-well plates (with 30,000 cells in 100 µL in each well) to which 1-µL volumes of compound dissolved in DMSO were added to final compound concentrations of 0.1, 0.25, 0.5, 1, 10, 25, 50, 75, and 100 µM. To control wells, 1 µL DMSO was added. Plates were incubated at 37 °C for 24 h. Then, they were centrifuged, the medium replaced by 100 µL of fresh medium without phenol red, and 50 µL of XTT-PMS mixture (50 µL XTT + 1 µL PMS) was added to each well. The plates were incubated for 4 hours at 37 °C and the absorbance at 450 and 500 nm recorded in an ELISA reader. Cell viability was calculated as explained.[35]

ASSOCIATED CONTENT

Supporting Information. Code, suppliers and molecular structures of all inhibitors tested. This material is available free of charge via the Internet at http://pubs.acs.org."

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

EPPS: 4-(2-Hydroxyethyl)-1-piperazinepropanesulfonic acid, 4-(2-Hydroxyethyl)piperazine-1-propanesulfonic acid; DMF: dimethylformamide; DCM: dichloromethane; XTT: tetrazolium salt; PMS: N-methylphenazonium methyl sulfate; DMSO: dimethyl sulfoxide; BHI: Brain Heart Infusion; VP-ITC: Pressure and Volume constant Isothermal Titration Calorimetry; OD: Optical Density; TMS: tetramethylsilane; TFA: trifluoroacetic acid; *p*-TSA: p-toluensulphonic acid; BR: biological response; TI: therapeutic index

REFERENCES

(1) Bruce, M. G., and Maaroos, H. I. Epidemiology of Helicobacter pylori infection. Helicobacter, **2008**, 13, 1-6.

(2) Marshall, B. J., and Warren, J. R. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet, **1984**, 1, 1311–1315.

(3) Malfertheiner, P., Megraud, F., O'Morain, C. A., Atherton, J., Axon, A. T., Bazzoli, F., Gensini, G. F., Gisbert, J. P., Graham, D. Y., Rokkas, T., El-Omar, E. M., and Kuipers, E. J. Management of Helicobacter pylori infection-the Maastricht IV/ Florence Consensus Report, Gut **2012,** 61, 646-664.

(4) Malfertheiner, P., Megraud, F., O'Morain, C., Bazzoli, F., El-Omar, E., Graham, D., Hunt, R., Rokkas, T., Vakil, N., and Kuipers, E. J. Current concepts in the management of Helicobacter pylori infection: the Maastricht III Consensus Report, Gut **2007,** 56, 772-781.

(5) Malfertheiner, P., Megraud, F., O'Morain, C., Bell, D., Bianchi Porro, G., Deltenre, M., Forman, D., Gasbarrini, G., Jaup, B., Misiewicz, J. J., Pajares, J., Quina, M., and Rauws, E. Current European concepts in the management of Helicobacter pylori infection--the Maastricht Consensus Report. The European Helicobacter Pylori Study Group (EHPSG), Eur J Gastroenterol Hepatol, **1997**, 9, 1-2.

(6) Martinez-Julvez, M., Rojas, A. L., Olekhnovich, I., Espinosa Angarica, V., Hoffman, P. S., and Sancho, J. Structure of RdxA--an oxygen-insensitive nitroreductase essential for metronidazole activation in Helicobacter pylori. FEBS J., **2012**, 279, 4306-4317.

(7) Secka, O., Berg, D. E., Antonio, M., Corrah, T., Tapgun, M., Walton, R., Thomas, V., Galano, J. J., Sancho, J., Adegbola, R. A., and Thomas, J. E. Antimicrobial Susceptibility and Resistance Patterns among Helicobacter pylori Strains from The Gambia, West Africa. Antimicrob Agents Chemother, **2013**, 57, 1231-1237.

(8) Fiorini, G., Zullo, A., Gatta, L., Castelli, V., Ricci, C., Cassol, F., and Vaira, D. Newer agents for Helicobacter pylori eradication. Clin Exp Gastroenterol, **2012**, 5, 109-112.

(9) Aebischer, T., Meyer, T. F., and Andersen, L. P. Inflammation, immunity, and vaccines for Helicobacter. Helicobacter **2010**, 15 Suppl 1, 21-28.

(10) Blaser, M. Antibiotic overuse: Stop the killing of beneficial bacteria. Nature, **2011**, 476, 393-394.

(11) Chalker, A. F., Minehart, H. W., Hughes, N. J., Koretke, K. K., Lonetto, M. A., Brinkman, K. K., Warren, P. V., Lupas, A., Stanhope, M. J., Brown, J. R., and Hoffman, P. S. Systematic identification of selective essential genes in Helicobacter pylori by genome prioritization and allelic replacement mutagenesis. J. Bacteriol., **2001**, 183, 1259-1268.

(12) Cremades, N., Bueno, M., Toja, M., and Sancho, J. Towards a new therapeutic target: Helicobacter pylori flavodoxin. Biophys. Chem., **2005**, 115, 267-276.

(13) Freigang, J., Diederichs, K., Schafer, K. P., Welte, W., and Paul, R. Crystal structure of oxidized flavodoxin, an essential protein in Helicobacter pylori. Protein Sci., **2002**, 11, 253-261.

(14) Sancho, J. Flavodoxins: sequence, folding, binding, function and beyond. Cell. Mol. Life Sci., **2006**, 63, 855-864.

(15) Hughes, N. J., Chalk, P. A., Clayton, C. L., and Kelly, D. J. Identification of carboxylation enzymes and characterization of a novel four-subunit pyruvate:flavodoxin oxidoreductase from Helicobacter pylori. J. Bacteriol., **1995**, 177, 3953-3959.

(16) St Maurice, M., Cremades, N., Croxen, M. A., Sisson, G., Sancho, J., and Hoffman, P. S. Flavodoxin:Quinone Reductase (FqrB): a redox partner of pyruvate:ferredoxin oxidoreductase that reversibly couples pyruvate oxidation to NADPH production in Helicobacter pylori and Campylobacter jejuni. J. Bacteriol., **2007**, 189, 4764-4773.

(17) Cremades, N., Velazquez-Campoy, A., Martinez-Julvez, M., Neira, J. L., Perez-Dorado, I., Hermoso, J., Jimenez, P., Lanas, A., Hoffman, P. S., and Sancho, J. Discovery of specific flavodoxin inhibitors as potential therapeutic agents against Helicobacter pylori infection, ACS Chemical Biology, **2009**, 4, 928-938.

(18) Kumaran, G., and Gurunath, H. K. Titanium (IV) chloride-triethylsilane mediated conversión of ω-nitrostyrenes to phenylacetohydroximoyl chlorides. , Tetrahedron Lett., **1994**, 35, 9099-9100.

(19) Ju-Tsung, L., and Ching-Fa, Y. One-pot synthesis of trans-β-alkylstyrenes. Tetrahedron Lett., **2001**, 42, 6147-6150.

(20) Wadsworth, W. S. Synthetic applications of phosphoryl-stabilized anions. Organic Reactions, **2005**, 73-253.

(21) Basavaiah, D., Rao, A. J., and Satyanarayana, T. Recent advances in the Baylis-Hillman reaction and applications, Chem. Rev., **2003**, 103, 811-892.

(22) Marson, C. M. Reactions of carbonyl compounds with (monohalo)methyleniminium salts (Vilsmeier reagents). Tetrahedron, **1992**, 48, 3959-3726.

(23) Balkrisna, S. B., Childers, W. E. J., and Pinnick, H. W. Oxidation of α,β-unsaturated aldehydes. Tetrahedron, **1981**, 37, 2091-2096.

(24) Verma, R. K., and Kumar, B. Esterification at room temperature: a mixing affair only. Synth. Commun., **1984**, 14, 1359-1363.

(25) Posner, G. H. Conjugate addition reactions of organocopper reagents. Organic Reactions, **1972**, 19, 1-113.

(26) Chaikin, S. W., and Brown, W. G. Reduction of aldehydes, ketones and acid chlorides by sodium borohydride. J. Am. Chem. Soc., **1949**, 71, 122-125.

(27) Larock, R. Comprehensive organic transformations. VCH: New York, **1989**, pp151-152.

(28) Larock, R. Comprehensive organic transformations. VCH: New York, **1989**, pp 981-985.

(29) Cosimelli, B., Roncucci, G., Dei, D., Fantetti, L., Ferroni, F., Ricci, M., and Spinelli, D. Synthesis and antimycotic activity of new unsymmetrical substituted zinc phthalocyanines. Tetrahedron, **2003**, 59, 10025-10030.

(30) Urquhart, G. G., Gates, J. W., and Connor, R. In Org. Syntheses Coll. , Vol. 3, John Willey: New York, **1955**, pp 363-365.

(31) Petersson, M. J., Jenkins, I. D., and Loughlin, W. A. The use of phosphonium anhydrides for the synthesis of 2-oxazolines, 2-thiazolines and 2-dihydrooxazine under mild conditions. Org. Biomol. Chem., **2009**, 7, 739-746.

(32) Choi, J. S., Lee, H.-S., Lee, Y., Jeong, N., Kim, H.-J., Kim, Y.-D., and Han, H. Nsc-mediated solid-phase synthesis of polyamides containing pyrrole amino acid. Tetrahedron Lett., **2002**, 43, 4295-4299.

(33) Hazlet, S. E., and Dornfeld, C. A. The reduction of aromatic nitro compounds with activated iron. J. Am. Chem. Soc., **1944**, 66, 1781-1782.

(34) Cremades, N., Velazquez-Campoy, A., Freire, E., and Sancho, J. The flavodoxin from Helicobacter pylori: structural determinants of thermostability and FMN cofactor binding. Biochemistry, **2008**, 47, 627-639.

(35) Lopez, L. C., Dos-Reis, S., Espargaro, A., Carrodeguas, J. A., Maddelein, M. L., Ventura, S., and Sancho, J. Discovery of novel inhibitors of amyloid beta-peptide 1-42 aggregation. J. Med. Chem., **2012**, 55, 9521-9530.

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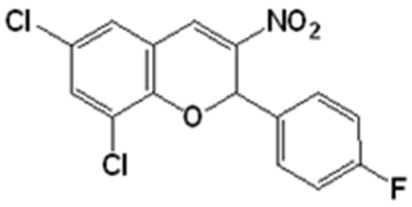

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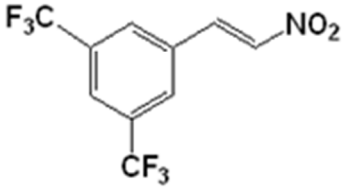

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