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CONCISE ARTICLE

# Tetrahydro-pyrimido-indoles as selective LIMK inhibitors: synthesis, selectivity profiling and structure-activity studies‡

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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Extensive structure-activity studies on three different modification sites resulted in a series of LIM kinase inhibitors, containing a novel tricyclic hinge-binding motif based on the pyrrolopyrimidine scaffold. The compounds display a superior selectivity profile and significantly increased on-target activity compared to the former clinical candidate LX7101 (Lexicon Pharmaceuticals). Additionally, a soft drug approach to yield locally active analogues was successfully implemented.

## Introduction

LIM kinases (LIMK)[1] are serine/threonine kinases, represented by two isoforms, LIMK1[2] and LIMK2,[3] that share 70% homology in the kinase domain. The main substrates of LIMK are cofilin1, cofilin2 and destrin,[4] though other potential protein substrates have been proposed as well. LIMK play a key role in the reorganization of the actin cytoskeleton,[5] and consequently in the regulation of cell morphology and motility, by inactivating members of the cofilin family through phosphorylation on Ser3. The activity of LIMK is regulated by several Rho GTPase family signalling pathways. Both Rho-associated coiled-coil containing kinase isoforms ROCK1 and ROCK2 have been shown to phosphorylate and activate LIMK. A similar effect has been observed for p21-activated kinases (PAK1, PAK2 and PAK4),[6] and myotonic dystrophy kinase-related Cdc42-binding kinase (MRCKα) as well. In fact, our interest in LIMK stems directly from our focus on ROCK and its inhibitors,[7] as ROCK is able to phosphorylate a conserved Thr of the LIMK kinase domain. Located downstream from ROCK, targeting LIMK might result in a comparable biological effect, while potentially reducing the side effects.[8] Phosphatases, such as slingshot 1 (SSH1) and chronophin (CIN) on the other hand, inactivate LIMK. In view of their influence on important cellular processes, such as cell morphology, motility and barrier integrity, it stands to reason that LIM kinases have been associated with several diseases, such as glaucoma/elevated intraocular pressure,[8,9] Williams syndrome,[10] intracranial aneurysms,[11] cancer,[6] pulmonary hypertension,[12] and even HIV infection.[13]

The amount of effort invested in unravelling the importance of LIM in cellular processes is, however, not reflected in the number of small molecule LIMK inhibitors reported to date, allowing them to be classified into merely five distinct series. Out of these, the two main series are based on either the 2-aminothiazole[14] or pyrrolopyrimidine[8,9,15,16] scaffold. Fewer publications have appeared on the three remaining series, represented by 4-aminobenzothienopyrimidines,[17] pyridocarbazolones[18] and arylsulfonamides[19] respectively.

## Results and discussion

### Kinase selectivity profiling

Recently,[16] we reported the design and synthesis of selective LIMK inhibitors based on the potent, yet not fully selective compound LX7101 (**1**, Lexicon Pharmaceuticals, Figure 1),[9] to the best of our knowledge the only LIMK inhibitor that has reached the clinical stage until now. We came to the conclusion that the pharmacological effect of LX7101 might be (at least partly) attributed to off-target effects, most notably ROCK inhibition. We demonstrated that 5,6-dimethyl substitution of the pyrrolopyrimidine (**2**, Figure 1) was key in inducing this level of selectivity for LIMK inhibition over ROCK2 and PKA inhibition in this series. Unfortunately, in-depth exploration of structure-activity relationships on these 5,6-dimethylpyrrolopyrimidine-based compounds was halted due to concerns regarding an already crowded IP space. Ring closure of the 5,6-dimethyl substituents of **2** towards the tricyclic derivative **3** provided an elegant way to circumvent these IP issues (Figure 1). Gratifyingly, the direct tricyclic analogue **3** exhibited the same degree of selectivity as its 5,6-dimethyl precursor **2**, combining low to subnanomolar on-target activity for LIMK (being 30 times more potent for LIMK2 than LIMK1) with a strong selectivity against both ROCK2 and PKA.

To further demonstrate the improved degree of selectivity of this novel kinase scaffold, the selectivity profiles of **1** (LX7101) and of its tricyclic counterpart **3** against a panel of 342 kinases (Reaction Biology Corp.) were compared. Both compounds

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Electronic Supplementary Information (ESI) available: See
DOI: 10.1039/x0xx00000x

were tested at 300 nM against a relatively low ATP concentration of 10 μM to identify potential side activities. Results are visualized in Figure 2 by a small molecule-kinase interaction map, providing a qualitative summary of selectivity, which immediately highlights the superiority of **3** over **1**. To facilitate a more quantitative evaluation of off-target kinase activity, a formal selectivity score S was calculated by dividing the number of kinases hit (>50% inhibition) by the total number of kinases tested.[20] **1** had a selectivity score $S_{(300nM)}$ of 0.10, with off-target activities located within the TK, CAMK and AGC kinase families. Off-target inhibition above 90% was only observed for three members of the AGC family, namely p70S6K, PKA and PRKX. In contrast, **3** had an improved selectivity score $S_{(300nM)}$ of 0.01, with no off-target inhibition above 90% and exhibiting only moderate inhibition of 2 kinases, i.e. SIK2 (CAMK family) and RSK4 (AGC family).

As it now had been established that cyclisation into the tricyclic scaffold conserved the selectivity of the 5,6-dimethyl analogue, we could focus our efforts on exploring the structure-active relationship with respect to LIMK activity by a stepwise classical medicinal chemistry approach, changing a single structural element in each step.

#### Chemistry

The compounds were synthesized in 4-7 steps according to our previously reported method,[16] by nucleophilic aromatic substitution of the appropriate arylchloride with a piperidine derivative, followed by amide coupling with a suitably substituted aniline derivative (Scheme 1). In case of a primary amine side chain, a Boc-protection/deprotection strategy was required (a more detailed scheme can be found in the supplementary information). The tricyclic arylchloride was prepared in 2 steps as described previously,[21] through Fischer indole synthesis starting from 6-hydrazino-4(1*H*)-pyrimidinone and the required cyclic ketone, followed by conversion of the carbonyl function into the desired imidoyl chloride moiety by reaction with phosphoryl chloride.

#### Structure-activity and structure-property relationships

Our efforts were initially focused on identifying the best amine functionalized side chain by evaluating the on-target activity against LIMK1 and LIMK2 (Table 1) of a series of analogues, which conserved the tricyclic heterocycle used in **3** (Het A, Scheme 1). In general, the tested compounds were more potent against LIMK2 than LIMK1 (by a factor of 20-100), which is consistent with literature data.[8,9] Removal of the methylene linker of **3** had no effect on LIMK1, or LIMK2 potency (**4**). Dimethylation of the primary amine function increased the potency against LIMK2, while having no effect on LIMK1 potency, both in the case of **3** (vs **5**) and **4** (vs **6**). This allowed us to favour the tertiary amines over the primary amines, but did not help us discriminate between the compounds with or without methylene linker. As no difference in potency for LIMK1 could be observed in this small test group, we decided to focus on LIMK2 as primary screen. To guide us in deciding on the amine side chain, we evaluated **3**-**6** in a Caco-2 permeability assay in the apical-basolateral (A-B) direction (Table 1). In first instance, permeability was evaluated using a pH gradient across the cell monolayer, mimicking the situation in the intestine of a slightly acidic pH (6.5) in the lumen of the small intestine and neutral pH (7.4) in the blood. The low permeability of **3** can be increased to medium values by either removing the methylene linker (**4**) or dimethylation of the amine function (**5**). A combination of the two results in a compound with high permeability (**6**). Secondly, experimental conditions without pH gradient were chosen, which minimizes proton-driven transport. A similar trend in permeability can be observed, with low A-B permeability for **3**, medium $P_{app}$ for **4** and **5**, and high permeability for **6**, which is in line with the calculated log D values at physiological pH. In view of these permeability data and the LIMK potency, **6** was chosen as the scaffold of choice to design and synthesize further analogues.

Next, we focused our attention on the arylamide part of the molecule (Table 2), as up till now, we had conserved the substitution pattern of Lexicon's clinical candidate LX7101,[9] with a carbamate moiety in *meta*-position. As a starting point, the 'simple' phenyl analogue (**7**) was chosen as reference and was subsequently substituted with fluorine in *para*-, *meta*-, and *ortho*-position respectively. *Para*-F substitution led to a minor decrease (2-fold) in potency (**8** vs **7**), which was restored in the case of *meta*-F (**9**). *Ortho*-F substitution (**10**) led to a clear loss in potency, which was even worsened by changing the fluorine to methyl (**11**), leading us to disfavour *ortho*-substitution. As we knew from previous experience that *para*-substitution was not preferred to achieve potent LIMK inhibitors,[16] only a limited number of such analogues were synthesized (**8**, **12**-**16**). In short, bulky substituents were not tolerated (*p*-NHAc, **14**) in this position and electron withdrawing substituents (through inductive as well as resonance effects) resulted in the lowest $IC_{50}$ values (**8** and **12**), but as none of the *para*-substituted compounds reached single-digit nanomolar on-target activity, only *meta*-substitution was explored further. A methyl group in *meta*-position was well tolerated (**17**), in contrast with a similar-sized, but electronically different trifluoromethyl (**18**), which resulted in a significant drop in potency, as was also the case for a cyano group (**19**). Ethers on the other hand, had hardly any effect on the potency (**20** and **21**), even in the case of a rather bulky isopropyl ether. Introducing a basic centre in the form of a bulky piperidin-1-ylmethyl substituent led to a minor (ca. 2-fold) loss in activity compared to the starting compound (**22** vs **7**). Adding an oxygen atom in the heterocycle (morpholin-4-ylmethyl, **23**), however, caused a significant decrease in potency. The impact of a methyl ester in *meta*-position was a small decrease in LIMK2 activity (**24**), which could be turned into a significant increase in potency by inserting a methylene linker between the phenyl ring and ester moiety (**25**). The beneficial effect of a carbonyl in β-position of the aromatic ring could be confirmed by the acetyl protected hydroxy derivative **26**, which exhibited similar subnanomolar potency (and is also reflected in the subnanomolar activity of



 

the carbamate **6**). A similar trend could be observed for a sulfonyl series, with slightly decreased potency for the α-sulfonyl (methylsulfonyl **27**) and a subnanomolar $IC_{50}$ value for the β-derivative (methanesulfonamide **28**).

Another option that was explored, was *meta*-substitution with a (hetero-)aromatic ring. Introducing a simple phenyl ring already resulted in a promising (ca. 6-fold) gain in potency (**29** vs **7**), which was even further increased when a nitrogen atom was incorporated, most notably in the case of pyridin-4-yl (**31**). Likewise, 5-membered heterocycles proved to have a beneficial effect on the potency, resulting in equally potent compounds exhibiting subnanomolar $IC_{50}$ values, with two- (1*H*-imidazolyl-1-yl **35** and 1*H*-pyrazol-3-yl **36**) and three (1*H*-1,2,4-triazol-1-yl **38**) nitrogen atom containing heterocycles being the most active ($IC_{50}$ values below 100 pM).

Lastly, the tricyclic kinase scaffold of compound **6** was altered (Table 3), using two other fused cycloalkanes (while retaining the $NMe_2$ amine side chain and *m*-carbamate substitution). As this could potentially lead to loss of selectivity, these compounds were counter-screened for ROCK2 activity as well. Changing to a cyclopentyl fused system (Scheme 1, Het B) resulted in a mild decrease (4-fold, **40** vs **6**) in LIMK2 potency, while difluoro-substitution (**41**; Scheme 1, Het C) had a negligible effect (2-fold). Selectivity *vs* ROCK2 and PKA, which was already acquired from a methyl substitution on position 6 of the pyrrolopyrimidine, was conserved for both fused ring systems. Similarly selectivity of selected compounds displaying a range of *meta* and *para* substitutions on the phenyl moiety was confirmed.[23] Bearing in mind that changing the ring size or introducing difluoro-substitution might also have an influence on the permeability, the 2 analogues were also evaluated in a Caco-2 assay. Both compounds retained high Caco-2 permeability (with a pH 6.5/7.4 A-B $P_{app}$ of 22 and 19 $10^{-6}$ $cms^{-1}$ for compounds **40** and **41** respectively).

#### Soft drug approach

Though it seems reasonable to assume that targeting LIMK (downstream of ROCK) would result in fewer (systemic) side effects than inhibiting ROCK itself,[8] there is hardly any data to support this claim. In view of this, we reasoned that it might be wise to investigate the option of a soft drug approach, i.e. locally active compounds (following topical application) that should be degraded into a predefined, functionally inactive metabolite after leaving the site of action to reduce systemic side effects.[22]

Following our experience with soft ROCK inhibitors,[7] our strategy was to use ester moieties to achieve this goal, as they can be easily hydrolysed into the corresponding carboxylic acids that are usually devoid of functional activity due to lower permeability.[24] To achieve a maximum of diversity, we focused on the three hinge-binding scaffolds discussed above, in combination with two different amine side chains, namely $NMe_2$ (which had been extensively used in the SAR studies) and $CH_2NH_2$ (for reasons of diversity, the side chain complementary to $NMe_2$ was chosen, with primary amine and methylene linker). Compounds were evaluated for their stability in human plasma, following a previously described protocol (Table 3).[7]

Compound **24** with $NMe_2$ as amine linker and cyclohexyl as fused ring, showed low nanomolar activity on LIMK2, but unfortunately, was stable in human plasma ($t_{1/2}$> 60 min). Simply changing the amine linker to $CH_2NH_2$ resulted in a slight drop in potency (ca. 2-fold, **42**), but also in lower plasma stability ($t_{1/2}$= 27 min). From our SAR studies, we deduced the benefit of shifting the carbonyl function to β-position by introducing a methylene linker. To our delight, the expected increase in potency to subnanomolar values was accompanied by the desired increased instability in human plasma (**25** vs **24**). Indeed, all phenyl acetic esters exhibited a plasma half-life below 10 minutes. Briefly, all compounds (**25**, **43**-**47**) belonging to this subseries followed the same trend, with higher potency for the $CH_2NH_2$ linker over the $NMe_2$ linker. The most potent compounds **43**, **45** and **47**, with a cyclohexl, cyclopentyl and difluorocyclohexyl fused ring system respectively, displayed a potency below 100 pM, which ranks them amongst our most active compounds to date. In combination with the required plasma stability profile, these data definitely open the door for a soft drug approach, wherever topical application is feasible. In this case, compounds with lower permeability (as for example observed with an $CH_2NH_2$ amine side chain) might even be preferred.

## Conclusions

We have described the design, selectivity profiling and extensive structure-activity studies (targeting three sites of modification) of a novel series of highly selective LIMK inhibitors, containing a tricyclic kinase scaffold. This work built on our former discovery that 5,6-dimethylsubstitution of the pyrrolopyrimidine scaffold yielded compounds with strong selectivity for LIMK. Cyclisation into a fused tricyclic scaffold allowed the exploration of novel chemical space, while fully retaining potency and selectivity. Selected compounds were also evaluated in terms of permeability in a Caco-2 assay under two different pH conditions. To anticipate the potential risk of systemic side effects, the feasibility of implementing a soft drug approach was briefly investigated. A suitable position for the introduction of ester groups, allowing compound hydrolysis in plasma, was identified. These results not only indicated that a soft drug approach is definitely a viable option for this novel series of selective LIMK inhibitors, but even led to compounds that rank amongst our most potent compounds to date, with LIMK2 $IC_{50}$ values that further improved over the clinical candidate LX7101. Further *in vitro* profiling of selected compounds, including evaluation of functional activity, is currently being undertaken and will be communicated in due time.

## Acknowledgements

We would like to thank the IWT-Flanders for financial support through O&O grant 120514.

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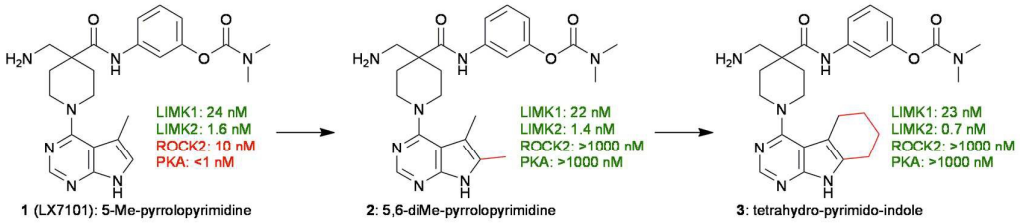



Figure 1: Schematic representation of the strategy to develop selective LIMK inhibitors, showing $IC_{50}$ values for 4 different kinases (at 1 µM ATP).

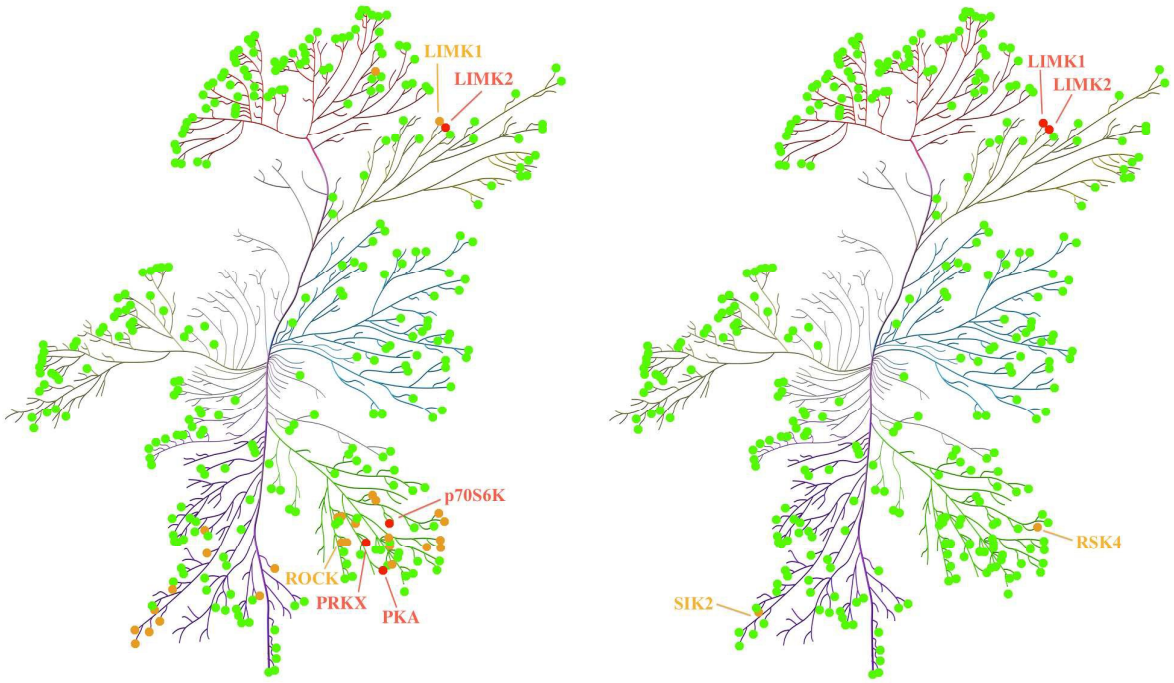
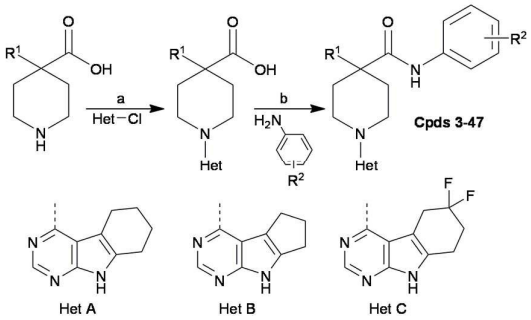
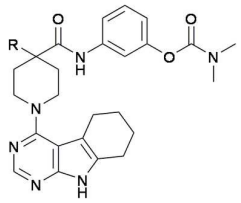



Figure 2: Kinase selectivity profile of **1** (left) and **3** (right), with red dots representing >90%, orange dots 50-90% and green dots <50% inhibition, respectively. Both compounds were tested at 300 nM against an ATP concentration of 10 µM.

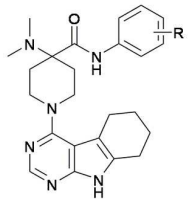



Scheme 1: Overview of compound synthesis. (a) DIPEA, DMSO/water (1:1), 140 °C, 16 h; b) T3P, DMAP, DCM, -15 °C, 16 h.



| Cpd | R | LIMK1 $IC_{50}$ (nM) | LIMK2 $IC_{50}$ (nM) | A-B $P_{app}$ ($10^{-6}$ cm/s) pH 6.5/7.4 | Calc. log D (pH 7.4) | A-B $P_{app}$ ($10^{-6}$ cm/s) pH 7.4/7.4 |
|---|---|---|---|---|---|---|
| **3** | $CH_2NH_2$ | 23 | 0.7 | 0.2 | 0.98 | <0.1 |
| **4** | $NH_2$ | 22 | 1.0 | 5.4 | 1.57 | 3.1 |
| **5** | $CH_2NMe_2$ | 20 | 0.2 | 7.7 | 1.22 | 0.5 |
| **6** | $NMe_2$ | 18 | 0.2 | 20 | 2.67 | 15 |

Table 1: Characterisation data for initial scaffolds **3**-**6** with variation on the amine side chain R.



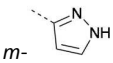
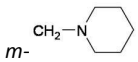
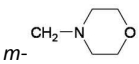
| Cpd | R | LIMK2 $IC_{50}$ (nM) | Cpd | R | LIMK2 $IC_{50}$ (nM) |
|---|---|---|---|---|---|
| **7** | H | 4.5 | **24** | *m*-COOMe | 13 |
| **8** | *p*-F | 12 | **25** | *m*-$CH_2$COOMe | 0.8 |
| **9** | *m*-F | 3.7 | **26** | *m*-OAc | 0.4 |
| **10** | *o*-F | 105 | **27** | *m*-$SO_2$Me | 12 |
| **11** | *o*-Me | 700 | **28** | *m*-$NHSO_2$Me | 0.4 |
| **12** | *p*-CN | 17 | **29** | *m*-  | 0.7 |
| **13** | *p*-$OCHF_2$ | 27 | **30** | *m*-  | 0.1 |
| **14** | *p*-NHAc | 122 | **31** | *m*-  | <0.1 |
| **15** | *p*-OMe | 41 | **32** | *m*-  | 0.2 |
| **16** | *p*-$NMe_2$ | 22 | **33** | *m*-  | 0.3 |
| **17** | *m*-Me | 7.1 | **34** | *m*-  | 0.3 |
| **18** | *m*-$CF_3$ | 31 | **35** | *m*-  | <0.1 |
| **19** | *m*-CN | 32 | **36** | *m*-  | <0.1 |
| **20** | *m*-OMe | 2.0 | **37** | *m*-  | 0.9 |
| **21** | *m*-O$^i$Pr | 4.8 | **38** | *m*-  | <0.1 |
| **22** | *m*-  | 11 | **39** | *m*-  | 0.1 |
| **23** | *m*-  | 62 | | | |

Table 2: SAR for compounds **7**-**39** with different substitution pattern on the phenyl ring of the arylamide.

| Cpd | $R^1$ | Het | $R^2$ | LIMK2 $IC_{50}$ (nM) | h plasma $t_{1/2}$ (min) |
|---|---|---|---|---|---|
| **40** | $NMe_2$ | Het B | *m*-$OCONMe_2$ | 0.9 | ND |
| **41** | $NMe_2$ | Het C | *m*-$OCONMe_2$ | 0.4 | ND |
| **24** | $NMe_2$ | Het A | *m*-COOMe | 13 | >60 |
| **42** | $CH_2NH_2$ | Het A | *m*-COOMe | 23 | 27 |
| **25** | $NMe_2$ | Het A | *m*-$CH_2COOMe$ | 0.8 | <10 |
| **43** | $CH_2NH_2$ | Het A | *m*-$CH_2COOMe$ | <0.1 | <10 |
| **44** | $NMe_2$ | Het B | *m*-$CH_2COOMe$ | 1.4 | <10 |
| **45** | $CH_2NH_2$ | Het B | *m*-$CH_2COOMe$ | <0.1 | <10 |
| **46** | $NMe_2$ | Het C | *m*-$CH_2COOMe$ | 0.2 | <10 |
| **47** | $CH_2NH_2$ | Het C | *m*-$CH_2COOMe$ | <0.1 | <10 |

Table 3: SAR and human plasma stability data for cpds **24**,**25**,**40**-**47** (Scheme 1); ND: not determined.

## Graphical abstract