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Asymmetric synthesis of the four diastereoisomers of a novel non-steroidal farnesoid X receptor (FXR) agonist: Role of the chirality on the biological activity

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ABSTRACT

An asymmetric synthetic strategy was designed for the preparation of the four possible diastereoisomers of 3,6-dimethyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one, a non-steroidal FXR agonist, we recently discovered following a virtual screening approach. The results obtained from an AlphaScreen assay clearly demonstrated that only the isomer endowed with 4*R*,6*S* absolute configuration is responsible for the biological activity. A deep investigation of the different putative binding modes adopted by these enantiomerically pure ligands using computational modeling studies confirmed the enantioselectivity of FXR towards this class of molecules.

1. Introduction

Starting from 1999 when the orphan nuclear receptor farnesoid X receptor (FXR) was 'de-orphanized' by the demonstration that primary bile acids (BAs) were its endogenous ligands,[1–3] great progress has been made in the understanding of its physiological roles. FXR was initially identified as a regulator of the expression of various transport proteins and biosynthetic enzymes that maintain cholesterol and bile acid homeostasis.[4] Indeed, activation of FXR by bile acids or synthetic agonists results in (a) transcriptional repression of cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in the bile acid biosynthesis pathway, (b) induction of the small heterodimer partner (SHP), a transcriptional repressor found in the liver and intestine, and (c) induction of genes encoding for some bile acid transport proteins, such as intestinal bile acid-binding protein (IBABP)[5] and bile salt export pump (BSEP).[6] Furthermore, bile acid-mediated FXR activation has been recently recognized as a major underlying pathway for energy homeostasis and glucose and lipid metabolism.[7] More recently, additional functional roles of FXR have been identified; specifically, it has been shown that FXR regulates normal liver regeneration,[8] plays a protective role in liver carcinogenesis and protects against intestinal infection.[9] All these evidences make FXR a promising potential target for the treatment of a variety of pathological conditions, including steatohepatitis, liver fibrosis, cholestasis, liver cancer, inflammatory bowel disease, cholesterol gallstone disease, atherosclerosis, erectile dysfunction, obesity, diabetes, and metabolic syndrome.[10–13]

For all these evidences, over the last decade, many efforts have been dedicated to the search of FXR small molecule modulators either by the structural modification of the endogenous ligands or by the screening of non-steroidal compound libraries.[14] Following the first approach, our group reported in 2002, 6α-ethylchenodeoxycholic acid (obeticholic acid, INT-747, **1**) as a highly potent and orally available FXR full agonist.[15] Positive data from two phase II clinical trials using INT-747 (**1**), recently demonstrated the clinical utility of this compound in the treatment of primary biliary cirrhosis, a chronic inflammatory cholestatic condition in the liver, and type 2 diabetes. The first non-steroidal FXR agonist, namely GW4064 (**2**), was identified by GlaxoSmithKline from an iterative combinatorial library synthesis and screening approach.[16] A lot of work was then dedicated to further explore structure/activity relationships (SAR) of **2** and improve its drug developability.[17] High-throughput screening combined with optimization of benzopyran-based combinatorial library result in 2003 in the discovery

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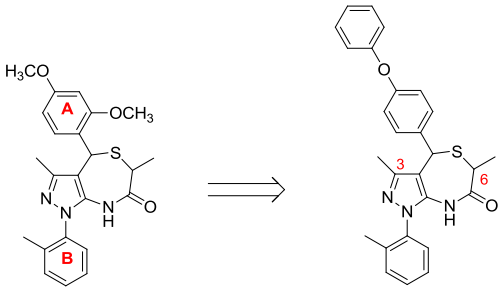
of fexaramine (**3**), another structurally diverse non-steroidal FXR agonist.[18] Aside from mentioned GW4064 (**2**) and fexaramine (**3**), high-throughput screening campaigns revealed benzimidazole- (**4**)[19] and azepino(4,5-*b*)indole (**5**)[20] derivatives as new classes of FXR agonists, while pyrazoline-3,5-dione- (**6**)[21] and 1-(4-methylpiperazin-1-yl)-3-phenoxypropan-2-ol (**7**)[22] derivatives were disclosed by virtual screening approaches (Fig. 1).

As a part of a project aimed at finding a novel class of non-steroidal FXR agonists we recently reported the identification of 4-(2,4-dimethoxyphenyl)-3,6-dimethyl-1-(2-mehylphenyl)-4,8-dihydro-1*H*-pyrazole[3,4-*e*][1,4]thiazepin-7-one (**8**), as a new scaffold endowed with FXR activity. Our first optimization work focused to explore substitution patterns of A and B rings of **8**, revealed that, among the two diastereoisomeric couples, the *syn* one is responsible for the biological activity. Similar indications arose also from the evaluation of the binding energies correlated to a set of docking studies, that confirmed the different putative binding pose of the two diastereoisomeric couples.[23] Having thus become aware that the relative disposition of the substituents is a crucial parameter for the activity and in consideration of the fact that the receptor recognition is expected to be enantioselective, we considered important to evaluate the activity of the single enantiomers of a target compound. With this aim, herein we report the synthetic approach allowing us to obtain the four possible diastereoisomers of 3,6-dimethyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one (**9**),[23] one of the most potent derivatives obtained from the optimization of **8**. Furthermore, as a continuation of the SAR study of this class of molecules, the synthesis and the biological evaluation of a series of analogues of **9** modified at C-3 and C-6-core positions will be described.



8 **9**

2. Chemistry

Although the preparation of functionalized 1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-ones is known,[24] there are no examples in the literature reporting the preparation of these compounds in enantiomerically pure form. The general synthetic strategy provides for the condensation reaction between a 5-aminopyrazole and an aldehyde, followed by the addition of the appropriate 2-mercaptocarboxylic acid to the intermediate 2,4-dihydro-3*H*-pyrazol-5-imine, first formed (Scheme 1). Since two stereogenic centers are present in the structure of the final compounds (if $R_3 \neq H$), they

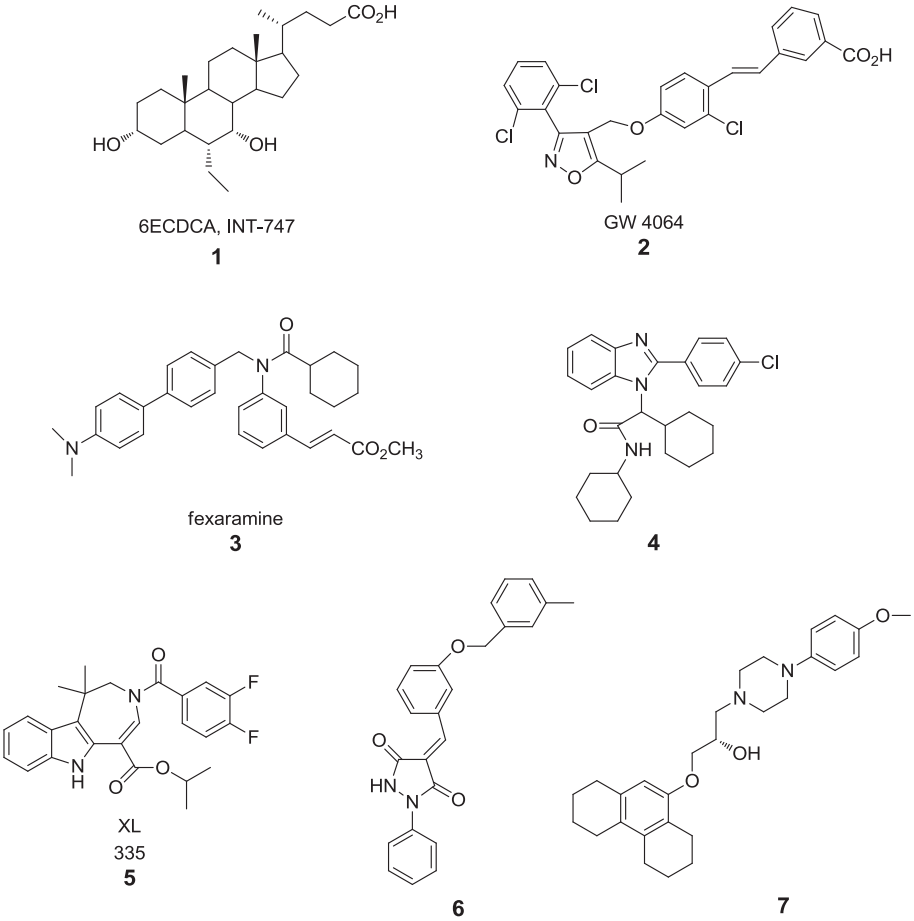
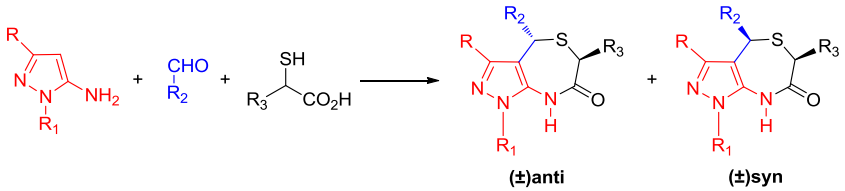


Figure 1. Structures of selected steroidal- and non-steroidal FXR agonists.



Scheme 1. General synthesis of functionalized 1*H*-Pyrazolo[3,4-*e*][1,4]thiazepin-7-ones.

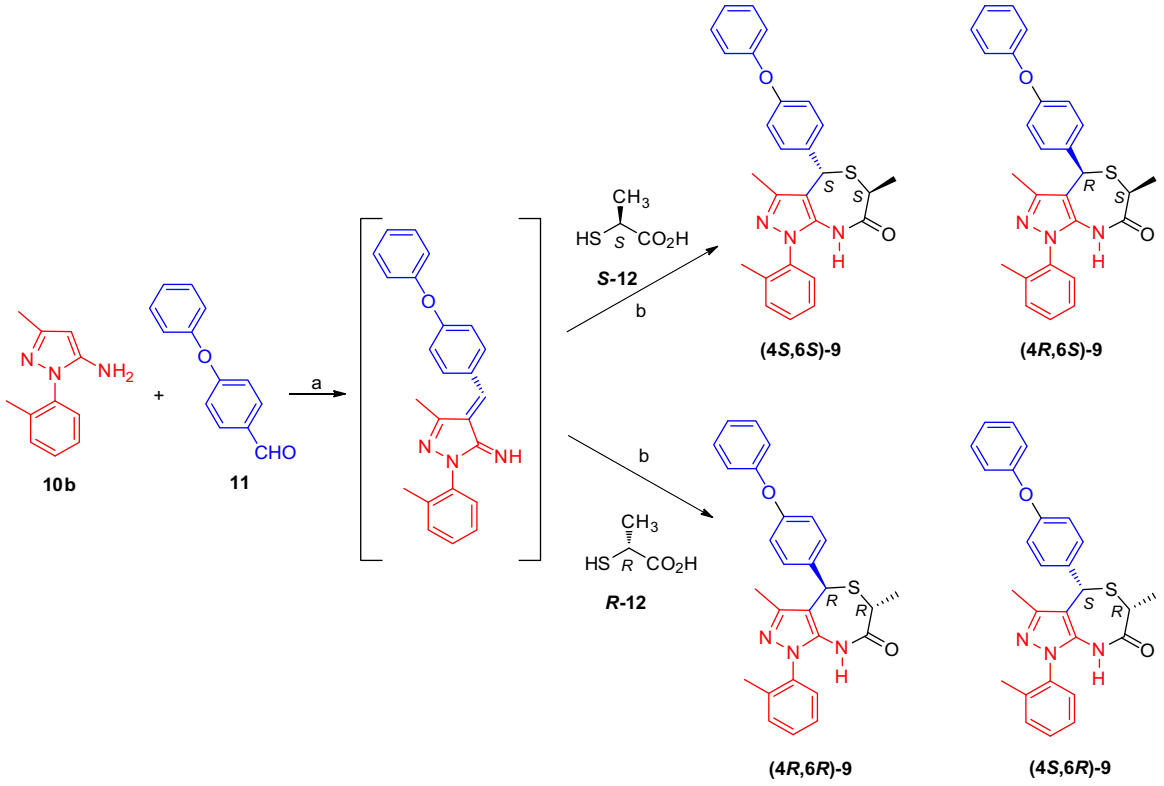
will be obtained as mixture of two racemic *anti*- and *syn*-couples, separable by chromatography.

By analyzing this reaction we realized that, while the asymmetric center at C-4 is formed during the reaction, the C-6 one derives from the starting 2-mercaptocarboxylic acid and remains intact during the reaction. Thus, it could be supposed that the use of a chiral, non-racemic 2-mercaptocarboxylic acid, in place of the corresponding racemate, should afford, after chromatography, the *anti*- and *syn* enantiomerically pure diastereoisomers. By repeating the reaction using the acid endowed with the opposite absolute configuration, it would then be obtained the other two diastereoisomers.

Thus, the addition of (*S*)-2-mercaptopropanoic acid [(*S*)-**12**] to the adduct derived from the condensation between 3-methyl-1-(2-methylphenyl)-1*H*-pyrazol-5-amine (**10b**) and 4-phenoxybenzaldehyde (**11**) afforded the mixture of the two 6*S*-diastereoisomers, which were separated by medium pressure chromatography (MPC) (Scheme 2). As detailed in our previous paper,[23] spectroscopic analysis allowed us to assign the *anti* disposition of the substituents to the first eluted compound, thus endowed with (4*S*,6*S*) absolute configuration and the *syn* (4*S*,6*R*) one to the more polar diastereoisomer. The preparation of the other diastereoisomeric pair, (4*R*,6*R*)-**9** and (4*S*,6*R*)-**9**, was achieved in the same fashion by using (*R*)-2-mercaptopropanoic acid [(*R*)-**12**] in the condensation step (Scheme 2). After MPC, (4*R*,6*R*)-**9** and (4*S*,6*R*)-**9** were obtained in 37% and 36% yield, respectively.

Although (*S*)- and (*R*)-2-mercaptopropanoic acids [(*S*)- and (*R*)-**12**] were synthesized according to the procedure reported by Kellogg and co-workers,[25] we deemed advisable to determinate their optical purity, not by the reported NMR methods, but by chiral HPLC, that is the same methodology we use for checking the enantiomeric purity of the final compounds. A series of unsuccessful analyses carried out on the racemate of 2-mercaptopropanoic acid (**12**) prompted us to attempt the enantioseparation of the corresponding *S*-trityl derivative considering the aromatic moiety able to emphasize the enantiodifference into an anisotropic environment.

A profitable enantioresolution ($\alpha = 1.33$, $R_S = 2.31$) of the racemate of *S*-trityl-2-mercaptopropanoic acid was achieved by running the analysis in the polar-organic mode of elution, with an anion-exchange chiral stationary phase based on quinine-carbamate chiral selector units (QN-AX CSP).[26] The chromatographic trace of the racemic mixture along with those referring to the (*R*)- and (*S*)-*S*-trityl-2-mercaptopropanoic acids are shown in



[a] (a) *i*. toluene, reflux; (b) *i*. reflux; *ii*. MPC.

Scheme 2. Synthesis of (4*S*,6*S*)-, (4*R*,6*S*)-, (4*R*,6*R*)- and (4*S*,6*R*)-3,6-Dimethyl-1-(2-methylphenyl)–4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-ones [(4*S*,6*S*)-**9**, (4*R*,6*S*)-**9**, (4*R*,6*R*)-**9** and (4*S*,6*R*)-**9**]. Reagents and conditions: (a) (i) toluene, reflux; (b) (i) reflux; (ii) MPC.

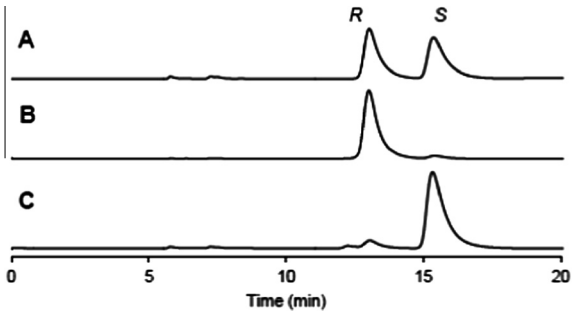
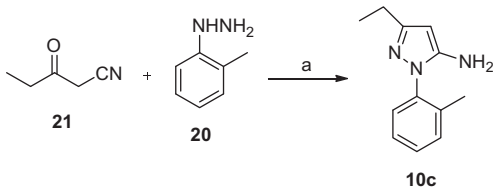


Figure 2. Chromatographic traces of (A) racemic S-Trityl-2-mercaptopropanoic acid; (B) S-Trityl-(R)-2-mercaptopropanoic acid and (C) S-Trityl-(S)-2-mercapto-propanoic acid. Mobile phase composition: MeOH/AcOH—99:1 (v/v).



[a] (a) *i*. EtOH abs, reflux, 12 h, 50 %.

Scheme 5. Synthesis of 3-Ethyl-1-(2-methylphenyl)-1*H*-pyrazol-5-amine (**10c**). Reagents and conditions: (a) (i) EtOH abs, reflux, 12 h, 50%.

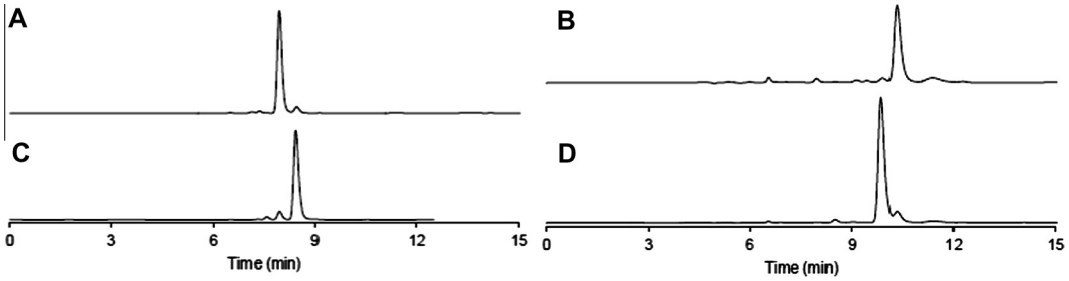
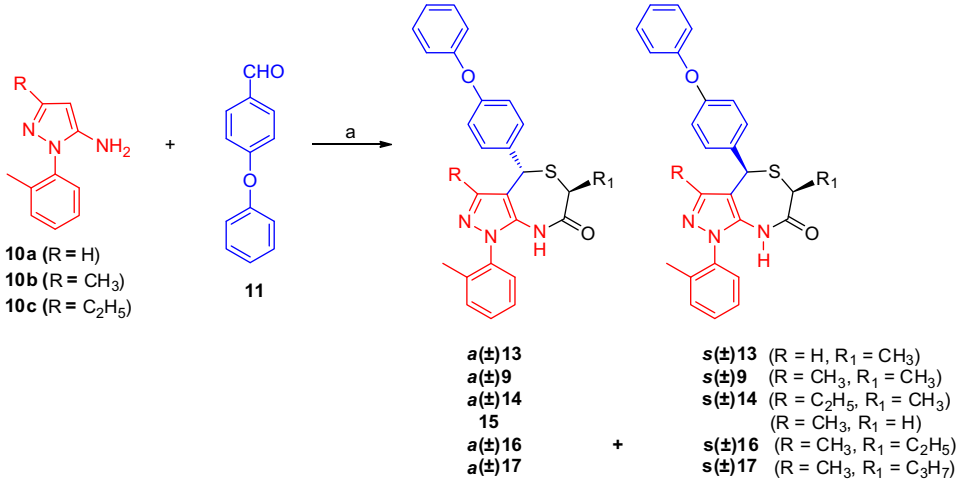
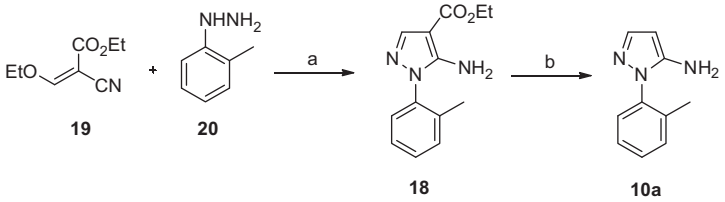


Figure 3. Chromatographic trace of (A) (4*R*,6*R*)-**9**; (B) (4*S*,6*R*)-**9**; (C) (4*S*,6*S*)-**9** and (D) (4*R*,6*S*)-**9**. Mobile phase composition: *n*-hexane/chloroform/EtOH—88:10:2 (v/v/v).



Scheme 3. Synthesis of 1*H*-Pyrazolo[3,4-*e*][1,4]thiazepin-7-ones, **a(±)**- and **s(±)9**, **13–17**. Reagents and conditions: (a) (i) toluene, reflux; (ii) thioglycolic acid (for **15**), 2-mercaptopropanoic acid (for **9**, **13**, **14**), 2-mercaptobutanoic acid (for **16**), 2-mercaptopentanoic acid (for **17**), toluene, reflux; (iii) MPC.



[a] (a) *i*. EtOH abs, reflux, 24 h, 56 %; (b) 12 N HCl, reflux, 12 h, 75 %.

Scheme 4. Synthesis of 1-(2-Methylphenyl)-1*H*-pyrazol-5-amine (**10a**). Reagents and conditions: (a) (i) EtOH abs, reflux, 24 h, 56%; (b) 12 N HCl, reflux, 12 h, 75%.

Table 1
FXR functional activity for (4*S*,6*S*)-, (4*R*,6*S*)-, (4*R*,6*R*)- and (4*S*,6*R*)-3,6-Dimethyl-1-(2-methylphenyl)–4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7(6*H*)-ones [(4*S*,6*S*)-**9**, (4*R*,6*S*)-**9**, (4*R*,6*R*)-**9** and (4*S*,6*R*)-**9**]

Compound	EC_{50}[a] (μM)	Efficacy[b] (%)
(4*S*,6*S*)-**9**	>150	
(4*R*,6*S*)-**9**	1.4 ± 0.2	130 ± 5
(4*R*,6*R*)-**9**	>150	
(4*S*,6*R*)-**9**	>150	

[a] Ligand-dependent recruitment of Src-1 peptide assessed by AlphaScreen assay. hFXR-LBD-GST was incubated with increasing concentrations of the indicated ligand in the presence of biotinylated Src-1 peptide. The AlphaScreen signal increases when the complex receptor-co-activator is formed.[31]
[b] Efficacy: % of compound effect versus 10 μM of CDCA.

Figure 2. Enantiomeric excess (ee) values of 85% and 90% were computed for the (*S*)-isomer and the (*R*)-isomer, respectively.

Instead, a cellulose-based CSP in combination with a 'non-standard' mobile phase system,[27] given the presence of chloroform, succeeded in the enantioresolution of the four diastereoisomers, (4*R*,6*R*)-**9**, (4*S*,6*R*)-**9**, (4*R*,6*S*)-**9** and (4*S*,6*S*)-**9**, which showed an ee of 87%, (Fig. 3A), 90% (Fig. 3B), 85% (Fig. 3C) and 80% (Fig. 3D), respectively ($\alpha = 1.14$, $R_S = 1.89$, for (4*R*,6*R*)-**9** and (4*S*,6*S*)-**9**; $\alpha = 1.07$, $R_S = 1.11$, for (4*R*,6*S*)-**9** and (4*S*,6*R*)-**9**). These values clearly indicate that the enantiomeric purity of the starting 2-mercaptopropanoic acid maintained nearly unaltered in the course of the synthetic procedures.

With the aim to explore the effects of modifications at C-3 and C-6 positions of **9**, following the synthetic protocol above described, 1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one derivatives **13–17** were also prepared (Scheme 3). Accordingly, the condensation reaction between the appropriate 1-(2-methylphenyl)-1*H*-pyrazol-5-amine **10** and 4-phenoxybenzaldehyde (**11**), carried out in refluxing toluene with concomitant water removal, and

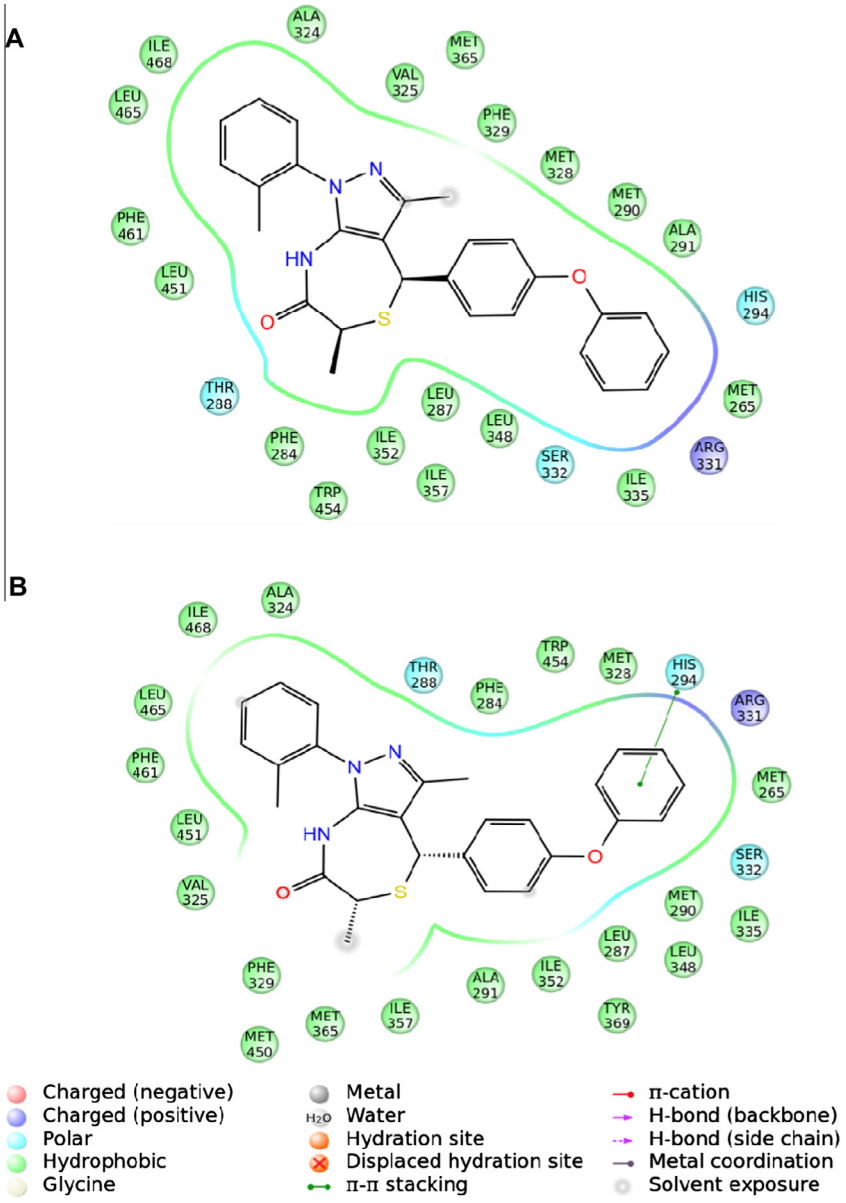
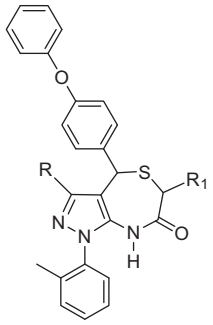


Figure 4. Ligand interaction diagrams of the best ranked docking poses of (A) the inactive *syn* enantiomer (4*S*,6*R*)-**9** in comparison (B) to the active one (4*R*,6*S*)-**9**. The interacting residues around the 1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one scaffold are completely different due to an opposite orientation of the core of these two ligands in the FXR LBD (1OSV pdb[32] residue numbering).

Table 2
FXR functional activity and calculated binding energies for dihydro-1*H*-Pyrazolo[3,4-*e*][1,4]thiazepin-7-ones, **9**, **13–17**



9, 13-17

Compound	R	R_1	EC_{50} (μM)[c]	Efficacy (%)[d]	ΔΔ*G* Binding solv. (kcal/mol)[e]	ΔΔ*G* Binding lipo (kcal/mol)[e]
***s*(±)9**[a]	CH_3	CH_3	3.0 ± 1.0	150 ± 30	Reference	Reference
***s*(±)13**[a]	H	CH_3	>150		+6.01	+8.44
***s*(±)14**[a]	CH_2CH_3	CH_3	2.0 ± 0.1	140 ± 50	−5.87	+7.10
15	CH_3	H	14	8	+4.01	+6.23
16[b]	CH_3	CH_2CH_3	3.0 ± 0.6	35 ± 8	+5.95	+1.51
***s*(±)17**[a]	CH_3	$CH_2CH_2CH_3$	>150		+6.64	+9.40

[a] The symbol *s* refers to the racemic couple endowed with *syn*-disposition of the substituents at C-4 and C-6 positions. The *anti*-couples resulted in any case less active than the corresponding *syn*-couples. Data relative to the *anti*-couples are not shown.
[b] Compound tested as mixture of *syn*- and *anti*-couples.
[c] Ligand-dependent recruitment of Src-1 peptide assessed by AlphaScreen assay. hFXR-LBD-GST was incubated with increasing concentrations of the indicated ligand in the presence of biotinylated Src-1 peptide. The AlphaScreen signal increases when the complex receptor–coactivator is formed.[31]
[d] Efficacy: % of compound effect versus 10 μM of CDCA.
[e] Difference of the binding energies estimated by Prime *MM-GBSA* calculation using the (4*R*,6*S*)-**9** values as the reference ones. In particular the Δ*G* binding solv. (desolvation) and Δ*G* binding lipo (lipophylic) contributes are reported.

the subsequent addition of the appropriate 2-mercaptocarboxylic acid afforded the title compounds **13–17**, obtained as mixture of two racemic couples (with the exception of the derivative **15** presenting only one stereogenic center). MPC of the crude reaction mixtures allowed us to obtain the less polar racemic couples ***a*(±)13–17**, endowed with an *anti* disposition of the substituents and the more polar ones, ***s*(±)13–17** showing a *syn* disposition of the substituents.[23] The separation was not possible for the compound **16** which was therefore tested as a mixture of four diastereoisomers.

The precursor 5-aminopyrazole **10b**, characterized by the presence of the methyl group at C-3 position, was prepared in high yield following a known procedure involving the reaction of 3-aminocrotononitrile with 2-tolylhydrazine in concentrated hydrochloric acid.[28] 1-(2-Methylphenyl)-1*H*-pyrazol-5-amine (**10a**) was synthesized (Scheme 4) by acidic hydrolysis and decarboxylation from ethyl 5-amino-1-(2-methylphenyl)-1*H*-pyrazole-3-carboxylate (**18**), in turn obtained by the condensation reaction between ethyl 3-ethoxy-2-cyanoacrylate (**19**) and 2-tolylhydrazine (**20**).[29] Whereas the condensation between the same hydrazine **20** and 3-oxopentanenitrile (**21**)[30] allowed us to obtain 3-ethyl-substituted pyrazole **10c** (Scheme 5). With regard to 2-mercaptocarboxylic acids, whereas thioglycolic and 2-mercaptopropanoic acids are commercially available, 2-mercaptobutanoic and 2-mercaptopentanoic acids were prepared from the corresponding 2-bromoacids by treatment with cesium thiobenzoate followed by aminolysis, according to the procedure followed by us for the preparation of (*S*)-and (*R*)-2-mercaptopropanoic acids [(*S*)- and (*R*)-**12**].[25]

3. Results and discussion

The ability of the synthesized compounds to act as FXR agonists was evaluated by ligand-dependent recruitment of steroid receptor coactivator-1 (SRC-1) peptide assessed by AlphaScreen assay.[31] The data relating to the four diastereoisomers (4*S*,6*S*)-, (4*R*,6*S*)-, (4*R*,6*R*)- and (4*S*,6*R*)-**9**, reported in Table 1, clearly demonstrated that the recognition process of the receptor towards our ligands is highly enantioselective. Only the diastereoisomer endowed with 4*R*,6*S* absolute configuration, indeed, was able to activate FXR receptor with a potency of 1.4 μM and a full efficacy. The absolute configuration of the active isomer (4*R*,6*S*)-**9** corresponds to a *syn* disposition of the substituents confirming what we have already observed on all derivatives of this class, with the *syn* couples resulting in any case more active than the corresponding *anti*-ones.[23]

With the aim to interpret these biological results, we applied some computational docking experiments followed by more accurate rescoring estimating the binding energies of the compounds. As reported in our previous work,[23] the pdb used (code: 1OSV)[32] in the structure based approach applied to this series of derivatives was chosen between a set of three published complexes, comprising also the pdb entries 3BEJ[33] and 3DCT,[17a–c] using an ensemble docking technique. By the docking program present in the Schrodinger Suite 2012,[34] a Glide 5.5 run in single precision mode (*GlideScore SP*) was instrumental to collect the ten best ranked poses for each molecule. The docking experiments carried out on the active derivative (4*R*,6*S*)-**9**, and its enantiomer (4*S*,6*R*)-**9**, highlighted their different putative binding pose in the LBD of FXR. The best ranked solutions of each compound are presented in the Ligand interaction diagrams pictures (Fig. 4) maintaining fixed the orientation of the bicyclic core of the molecules (3D-docking images are available in the Supplementary data). It's noteworthy that the interacting residues around the 1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one region are completely different, thus indicating an opposite orientation of the core of the two enantiomers in the binding site. By comparing the binding energies calculated using the *Prime MM-GBSA*[34] protocol on all the ten collected poses, and analyzing the energetically best solutions, we estimated a loss of lipophilic

contacts (ΔG binding lipo) of 5.20 kcal/mol and of 11.57 kcal/mol in terms of desolvation energy (ΔG binding solv)[35] for the inactive (4*S*,6*R*)-**9** in comparison to the active (4*R*,6*S*)-**9**. Moreover, it is important to note that (4*S*,6*R*)-**9** was unable to reproduce the orientation of its enantiomer (4*R*,6*S*)-**9** in all the ten poses analyzed. These results are in line with the results of the biological evaluations and confirms the crucial role of the hydrophobic interactions in the LBD of FXR.

As evidenced from the data reported in Table 2, the presence of a methyl- or an ethyl group [**s(±)14**] at C-3 position of the pyrazole seems to be essential for the activity of the corresponding compounds as the unsubstituted derivative ***s*(±)13** resulted inactive. An hydrophobic cleft in the ligand binding site, defined by PHE284, THR288, TRP454 and PHE461 becomes occupied by these alkyl groups at C-3 position, giving stabilizing Van der Waals interactions to the secondary structures, mainly AF-2 helix (residue range 461–468) and helix 3, that are directly involved in the co-activator recruitment process. Moreover, from a conformational point of view, the substituent at C-3 position seems to be instrumental to allow the correct orientation of the phenoxyphenyl moiety inside the receptor. By the aim of the computational studies, the sum of the estimated binding energies reported a loss of 1.23 kcal/mol for the (4*R*,6*S*)-**14** and of 14.45 kcal/mol for the (4*R*,6*S*)-**13** with respect to the (4*R*,6*S*) **9** (Table 2). Also the substituent at C-6 position of the seven-members ring has a significant influence on the activity of the corresponding compounds. The derivative **15**, not substituted at this position, indeed, showed an almost complete loss of potency in comparison to the reference compound ***s*(±)9**. The replacement of the methyl group of ***s*(±)9** by ethyl one, as in ***s*(±)16**, was more or less neutral in terms of EC_{50}, whereas resulted in a substantial decrease in efficacy. Also in this case the computational studies highlighted an overall loss of interaction energies of 10.24 kcal/mol and 7.46 kcal/mol for (4*R*)-**15** and (4*R*,6*S*)-**16** respectively, using the (4*R*,6*S*)-**9** as reference (Table 2). The introduction of bulkier alkyl group, such as *n*-propyl, resulted in the inactive derivative ***s*(±)17**. Also in this case, we observed from the docking studies, a modification of the orientation of both the aryl moieties of the molecules inside the receptor, changing the interaction networks displayed by the most active compound ***s*(±)9** that are plausible to explain the loss of potency (results shown in the Supplementary data). Indeed the (4*R*,6*S*)-**17** showed a gain in the interaction energies of 2.76 kcal/mol with respect to (4*R*,6*S*)-**9** compound (Table 2), but displayed a loss of the key interactions with PHE284 and TRP454, the above cited two residues localized in the region responsible for the co-activator recruitment.

4. Conclusion

In conclusion, the synthesis and the biological evaluation of the four possible diastereoisomers of the FXR full agonist 3,6-dimethyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one (**9**) allowed us to demonstrate that the receptor recognition is highly enantioselective for this class of agonists, as only (4*R*,6*S*)-**9** exhibited low micromolar range of potency and full efficacy, whereas the other three diastereoisomers resulted completely inactive. (4*S*,6*S*)-, (4*R*,6*S*)-, (4*R*,6*R*)- and (4*S*,6*R*)-**9** represent the first examples of enantiomerically pure 4,6-disubstituted 4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-ones, here prepared by the asymmetric version of a known multicomponent reaction using both optically pure 2-mercaptopropanoic acids. Moreover, a novel HPLC method for determining the enantiomeric excesses of these acids has been reported as a valid alternative to the known NMR one. With the aim to enlarge the SAR of this class of non-steroidal FXR agonists a series of derivatives of **9**, modified at C-3 and C-6 positions, has been also prepared and evaluated. The presence of a methyl or ethyl group at C-3 position of the core is essential for the activity of the corresponding compounds, whereas the methyl group is optimal at C-6 position, as the unsubstituted **15**, and bulkier alkyl-substituted derivatives **16** and **17** resulted less active then the reference compound **9**. The computational studies confirmed the observed biological results, giving an estimation of the binding energies of the putative binding poses, useful to understand the mechanism of action of these compounds in stabilizing the AF-2 helix region in a bioactive conformation able to recruit the co-activator. The new insights gained in this work will be instrumental to further optimize this series of non-steroidal FXR agonists.

5. Experimental section

5.1. Chemistry

Commercially available starting materials, reagents, and solvents were used as supplied. MPC was performed on Merck LiChroprep Si 60 Lobar columns. Flash chromatography was performed on Merck silica gel (0.040–0.063 mm). ^{1}H NMR spectra were recorded on a Bruker AC400 as solutions in $CDCl_3$. Chemical shifts were recorded in ppm (δ) downfield of tetramethylsilane. The spin multiplicities are indicated by the symbols s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and bs (broad). Melting points were determined by the capillary method on a Büchi 535 electrothermal apparatus and are uncorrected. All target compounds possessed acceptable purity as verified by HPLC (see Supporting information).

5.1.1. General procedure for the synthesis of 1*H*-Pyrazolo[3,4-*e*][1,4]thiazepin-7-ones 9, 13–17

The appropriate 1-(2-methylphenyl)-1*H*-pyrazol-5-amine **10a–c** (1.0 mmol) and 4-phenoxybenzaldehyde (**11**, 1.0 mmol) were heated under reflux in toluene (50 mL) for 7–9 h; during this period produced water was removed with the aid of a Dean–Stark apparatus. The reaction mixture was cooled, the proper 2-mercaptocarboxylic acid (1.0 mmol) was added and the mixture heated again at reflux for 12 hours. The solvent was removed in vacuo and the residue submitted to MPC. Elution by light petroleum–ethyl acetate mixtures afforded the desired compounds.

5.1.2. (4*S*,6*S*)-3,6-Dimethyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [(4*S*,6*S*)-9]

Light petroleum/ethyl acetate (80:20), yield 32%, white solid, mp 93–95 °C; $[\alpha]_D^{20}$ −18.5° (c 0.50, CH_2Cl_2); ee = 80% (HPLC); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 0.96 (d, *J* = 7.2 Hz, 3H, 6-CH_3), 1.97 (s, 3H, 2-CH_3), 2.14 (s, 3H, 3-CH_3), 3.22 (q, *J* = 7.2 Hz, 1H, 6-CH), 5.15 (s, 1H, 4-CH), 6.95–7.50 (m, 14H, aromatics and NH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 12.72, 16.34, 17.251, 36.23, 42.18, 105.04, 118.39, 119.27, 123.70, 127.51, 128.51, 129.29, 129.82, 130.58, 131.73, 135.10, 136.20, 136.38, 136.96, 147.68, 156.57, 156.86, 172.12; Anal. calcd for $C_{27}H_{25}N_3O_2S$: C, 71.18; H, 5.53; N, 9.22; O, 7.02; S, 7.04, found: C, 71.20; H, 5.54; N, 9.21; S, 7.02.

5.1.3. (4*R*,6*S*)-4-(4-Phenoxyphenyl)-3,6-dimethyl-1-(2-methylphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [(4*R*,6*S*)-9]

Light petroleum/ethyl acetate (80:20), yield 37%, white solid, mp 152–155 °C; $[\alpha]_D^{20}$ +43.3° (c 0.65, CH_2Cl_2); ee = 85% (HPLC); ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 1.46 (d, *J* = 7.2 Hz, 3H, 6-CH_3), 1.90 (s, 3H, 2-CH_3), 2.10 (s, 3H, 3-CH_3), 3.81 (q, *J* = 7.2 Hz, 1H, 6-CH), 5.63 (s, 1H, 4-CH), 6.95–7.41 (m, 14H, aromatics and NH); ^{13}C NMR

($CDCl_3$, 100 MHz) δ = 13.34, 15.24, 17.21, 39.00, 44.91, 108.47, 118.76, 119.14, 123.56, 127.29, 128.25, 129.59, 129.78, 130.32, 131.54, 133.72, 135.30, 135.46, 136.60, 148.09, 156.71, 157.08, 172.67; Anal. calcd for $C_{27}H_{25}N_3O_2S$: C, 71.18; H, 5.53; N, 9.22; O, 7.02; S, 7.04, found: C, 71.19; H, 5.52; N, 9.20; S, 7.01.

5.1.4. (4*R*,6*R*)-3,6-Dimethyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [(4*R*,6*R*)-9]

$[\alpha]_D^{20}$ +20.0° (c 0.60, CH_2Cl_2); ee = 87% (HPLC).

5.1.5. (4*S*,6*R*)-3,6-Dimethyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [(4*S*,6*R*)-9]

$[\alpha]_D^{20}$ −38.5° (c 0.40, CH_2Cl_2); ee = 90% (HPLC).

5.1.6. anti-(±)-6-Methyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [a(±)13]

Light petroleum/ethyl acetate (80:20), yield 25%, white solid, mp 189–192 °C; ^{1}H NMR (400 MHz, $CDCl_3$) 1.29 (d, *J* = 7.2 Hz, 3H, 6-CH_3), 2.12 (s, 3H, 2-CH_3), 3.50 (q, *J* = 7.2 Hz,1H, 6-CH), 5.40 (s, 1H, 4-CH), 6.73–7.50 (m, 15H, aromatics and NH); ^{13}C NMR (100 MHz, $CDCl_3$) 15.61, 17.27, 36.73, 41.98, 108.71, 118.49, 119.27, 123.68, 127.40, 128.12, 129.64, 129.83, 130.56, 131.71, 135.40, 136.46, 136.67, 140.00, 156.65, 157.06, 172.37; Anal. calcd for $C_{26}H_{23}N_3O_2S$: C, 70.72; H, 5.25; N, 9.52; O, 7.25; S, 7.26, found: C, 70.78; H, 5.28; N, 9.510; S, 7.22.

5.1.7. *syn*-(±)-6-Methyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [s(±)13]

Light petroleum/ethyl acetate (80:20), yield 10%, white solid, mp 179–182 °C; ^{1}H NMR (400 MHz, $CDCl_3$) 1.49 (d, *J* = 7.2 Hz, 3H, 6-CH_3), 2.07 (s, 3H, 2-CH_3), 3.72 (q, *J* = 7.2 Hz,1H, 6-CH), 5.58 (s, 1H, 4-CH), 6.73–7.50 (m, 15H, aromatics and NH); ^{13}C NMR (100 MHz, $CDCl_3$) 15.79, 17.19, 40.43, 45.87, 110.38, 118.69, 119.25, 123.61, 127.34, 128.16, 129.77, 130.51, 131.65, 134.07, 134.77, 135.41, 136.75, 140.26, 156.64, 157.35, 172.04; Anal. calcd for $C_{26}H_{23}N_3O_2S$: C, 70.72; H, 5.25; N, 9.52; O, 7.25; S, 7.26, found: C, 70.73; H, 5.26; N, 9.53; S, 7.25.

5.1.8. *anti*-(±)-3-Ethyl-6-methyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [a(±)14]

Light petroleum/ethyl acetate (80:20), yield 17%, white solid, mp 163–175 °C; ^{1}H NMR (400 MHz, $CDCl_3$) 1.16 (t, *J* = 7.5 Hz, 3H, CH_2CH_3), 1.30 (d, *J* = 7.2 Hz, 3H, 6-CH_3), 2.14 (s, 3H, 2-CH_3), 2.31 (m, 2H, CH_2CH_3), 3.23 (q, *J* = 7.2 Hz, 1H, 6-CH), 5.22 (s, 1H, 4-CH), 6.98–7.47 (m, 14H, aromatics and NH); ^{13}C NMR (100 MHz, $CDCl_3$) 12.09, 16.33, 17.24, 20.38, 36.13, 42.04, 104.29, 118.30, 119.24, 123.66, 127.47, 128.48, 129.30, 129.80, 130.45, 131.74, 135.37, 135.96, 136.78, 137.11, 152.41, 156.58, 156.81, 172.07; Anal. calcd for $C_{28}H_{27}N_3O_2S$: C, 71.61; H, 5.80; N, 8.95; O, 6.81; S, 6.83, found: C, 71.63; H, 5.81; N, 8.94; S, 6.80.

5.1.9. *syn*-(±)-3-Ethyl-6-methyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [s(±)14]

Light petroleum/ethyl acetate (80:20), yield 27%, white solid, mp 172–175 °C; ^{1}H NMR (400 MHz, $CDCl_3$) 1.12 (t, *J* = 7.5 Hz, 3H, CH_2CH_3), 1.47 (d, *J* = 7.2 Hz, 3H, 6-CH_3), 2.07 (s, 3H, 2-CH_3), 2.27 (m, 2H, CH_2CH_3), 3.79 (q, *J* = 7.2 Hz, 1H, 6-CH), 5.71 (s, 1H, 4-CH), 6.98–7.47 (m, 14H, aromatics and NH); ^{13}C NMR (100 MHz, $CDCl_3$) 12.20, 15.11, 17.22, 20.82, 38.82, 44.57, 108.05, 118.75, 119.09, 123.51, 127.18, 128.22, 129.53, 129.75, 129.91, 130.14, 131.49, 134.21, 135.17, 135.73, 136.69, 152.91, 156.74, 157.00, 172.97; Anal. calcd for $C_{28}H_{27}N_3O_2S$: C, 71.61; H, 5.80; N, 8.95; O, 6.81; S, 6.83, found: C, 71.62; H, 5.81; N, 8.93; S, 6.81.

5.1.10. (±)-3-Methyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [(±)15]

Light petroleum/ethyl acetate (80:20), yield 44%, white solid, mp 172–173 °C; ^{1}H NMR (400 MHz, $CDCl_3$) 2.00 (s, 3H, 2-CH_3), 2.20 (s, 3H, 3-CH_3), 3.33 (d, *J* = 15.4 Hz, 1H, 6-CH_a), 3.40 (d, *J* = 15.4 Hz, 1H, 6-CH_b), 5.31 (s, 1H, 4-CH), 7.03–7.45 (m, 14H, aromatics and –NH); ^{13}C NMR (100 MHz, $CDCl_3$) 12.77, 17.21, 31.72, 42.47, 105.83, 118.46, 119.34, 132.73, 127.50, 128.44, 129.46, 129.82, 130.59, 131.72, 135.11, 135.28, 135.54, 136.93, 147.86, 156.52, 157.11, 170.10; Anal. calcd for $C_{26}H_{23}N_3O_2S$: C, 70.72; H, 5.25; N, 9.52; O, 7.25; S, 7.26, found: C, 70.75; H, 5.33; N, 9.49; S, 7.24.

5.1.11. *anti*-(±)+*syn*-(±)-6-Ethyl-3-methyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [a(±)16+s(±)-16]

Light petroleum/ethyl acetate (80:20), yield 41%, white solid; ^{1}H NMR (400 MHz, $CDCl_3$) 0.60 (t, *J* = 7.3 Hz, 3H, CH_2CH_3, *anti*), 1.13 (t, *J* = 7.3 Hz, 3H, CH_2CH_3, *syn*), 1.46–1.52 (m, 2H, CH_2CH_3, syn), 1.66–1.74 (m, 2H, CH_2CH_3, *anti*), 1.91 (s, 3H, 2-CH_3, *anti*), 1.98 (s, 3H, 3-CH_3, *anti*), 2.10 (s, 3H, 2-CH_3, *syn*), 2.15 (s, 3H, 3-CH_3, *syn*), 2.92 (m, 1H, 6-CH, *anti*), 3.56 (t, *J* = 7.3 Hz, 1H, 6-CH, *syn*), 5.23 (s, 1H, 4-CH, *anti*), 5.78 (s, 1H, 4-CH, *syn*), 6.70–7.53 (m, 28H, aromatics and –NH, *anti* + *syn*); ^{13}C NMR (100 MHz, $CDCl_3$) 10.37, 11.92, 12.81, 13.34, 14.14, 17.24, 22.17, 22.52, 42.20, 43.50, 45.01, 46.05, 104.94, 108.53, 118.63, 118.74, 118.87, 119.09, 123.50, 127.24, 127.43, 128.23, 128.47, 129.46, 129.59, 129.75, 130.20, 130.42, 131.47, 131.65, 133.96, 135.37, 136.27, 136.48, 136.72, 147.72, 148.08, 156.67, 157.01, 171.97, 172.12; Anal. calcd for $C_{28}H_{27}N_3O_2S$: C, 71.61; H, 5.80; N, 8.95; O, 6.81; S, 6.83, found: C, 71.64; H, 5.83; N, 8.91; S, 6.81.

5.1.12. *anti*-(±)-6-*n*Propyl-3-methyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [a(±)17]

Light petroleum/ethyl acetate (80:20), yield 21%, white solid; mp 168–170 °C; ^{1}H NMR (400 MHz, $CDCl_3$) 0.99 (t, *J* = 7.1 Hz, 3H, CH_2CH_3), 1.67–1.54 (m, 4H, CH_2CH_2), 1.91 (s, 3H, 2-CH_3), 2.10 (s, 3H, 3-CH_3), 3.64 (t, *J* = 6.8 Hz, 1H, 6-CH), 5.65 (s, 1H, 4-CH), 6.78 (s, 1H, NH), 6.98–7.47 (m, 13H, aromatics); ^{13}C NMR (100 MHz, $CDCl_3$,) 13.38, 13.87, 17.19, 20.50, 31.17, 44.18, 45.11, 108.56, 118.80, 119.14, 123.55, 127.33, 128.26, 129.62, 129.79, 130.30, 131.55, 133.92, 135.37, 135.62, 136.53, 148.16, 157.08, 172.17; Anal. calcd for $C_{29}H_{29}N_3O_2S$: C, 72.02; H, 6.04; N, 8.69; O, 6.62; S, 6.63, found: C, 72.04; H, 6.06; N, 8.68; S, 6.61.

5.1.13. *syn*-(±)-6-*n*Propyl-3-methyl-1-(2-methylphenyl)-4-(4-phenoxyphenyl)-4,8-dihydro-1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-one [s(±)17]

Light petroleum/ethyl acetate (80:20), yield 23%, white solid; mp 163–165 °C; ^{1}H NMR (400 MHz, $CDCl_3$) 0.66 (t, *J* = 7.3 Hz, 3H, CH_2CH_3), 0.79–0.88 (m, 1H, $CH_2CH_aCH_3$), 1.21–1.28 (m, 1H, $CH_2CH_bCH_3$), 1.46–1.52 (m, 1H, $CH_aCH_2CH_3$), 1.83–1.93 (m, 1H, $CH_bCH_2CH_3$), 1.99 (s, 3H, 2-CH_3), 2.16 (s, 3H, 3-CH_3), 3.04 (t, *J* = 6.8 Hz, 1H, 6-CH), 5.22 (s, 1H, 4-CH), 6.78 (s, 1H, NH), 6.97–7.49 (m, 13H, aromatics); ^{13}C NMR (100 MHz, $CDCl_3$,) 12.81, 13.36, 17.28, 18.93, 31.01, 41.47, 42.27, 105.04, 118.85, 123.48, 127.46, 128.34, 128.54, 129.51, 129.80, 130.06, 130.49, 131.69, 133.36, 135.36, 136.31, 136.81, 147.77, 157.04, 172.26; Anal. calcd for $C_{29}H_{29}N_3O_2S$: C, 72.02; H, 6.04; N, 8.69; O, 6.62; S, 6.63, found: C, 72.03; H, 6.06; N, 8.67; S, 6.62.

5.2. HPLC analysis

The analytical HPLC measurements were made on a Shimadzu (Kyoto, Japan) LC-20A Prominence, equipped with a CBM-20A communication bus module, two LC-20AD dual piston pumps, a SPD-M20A photodiode array detector, and a Rheodyne 7725i injector (Rheodyne Inc., Cotati, CA, USA) with a 20 μL stainless steel loop. A LC solution software from Shimadzu allowed the manipulation of the chromatographic data. Column temperature was controlled through a Grace (Sedriano, Italy) heater/chiller (Model 7956R) thermostat.

The chiral stationary phase employed for the enantioseparation of *S*-trityl-2-mercaptopropanoic acids was a prototype of the commercially available Chiralpak QN-AX column (from Chiral Technologies Europe, Illkirch, France) (120 Å pore size, 5 μm particle diameter and column dimension of 150 × 4 mm I.D.) which was kindly provided by Professor Wolfgang Lindner. Instead, the column employed for the enantioseparation of **9** was the commercially available Chiralpak IB (from Chiral Technologies, West Chester, PA, USA) (250 mm × 4.6 mm I.D., containing cellulose tris(3,5-dimethylphenylcarbamate) immobilized onto a 5 μm silica gel).

Before the use, all the employed mobile phases were degassed through sonication. Analytes to be injected were solubilized into the selected mobile phase. The Chiralpak QN-AX column was conditioned with the selected mobile phase at a 1.0 mL min^{-1} flow rate for 20 min, before running the analysis; the Chiralpak IB column for at least 40 min.

The detection wavelength was always set at 254 nm. Unless otherwise stated, all the analyses were carried out at a 0.7 mL min^{-1} eluent flow rate, while the column temperature fixed at 25 °C.

All the following chromatographic parameters were calculated according to the German Pharmacopoeia (DAB). The retention factor (k) values were computed by taking the retention time (t_R) at the peak maximum. Enantioseparation factor (α), resolution factor (R_S) were computed from the following Equations 1 and 2:

$$\alpha = \frac{k_2}{k_1} \quad (1)$$

$$R_S = 1.18\frac{t_R - t_{Rp}}{W_{0.5} + Wp_{0.5}} \quad (2)$$

where k^1 is the retention factor of the first eluted enantiomer, k^2 is the retention factor of the second eluted enantiomer.

5.3. Docking and modeling studies

A structure based procedure was applied to discover and study the putative binding mode of the most potent compounds in the series. The pdb used (code: 1OSV[32]) was firstly identified in our previous work[23] between a set of three published complexes comprising also the pdb entries 3BEJ[33] and 3DCT[17a–c] using an ensemble docking technique. All the PDBs used in this work were previously submitted to the *Protein Preparation Wizard* protocol of the *Schrödinger Suite*. All the compounds were prepared by the aim of the *LigPrep* protocol present in the *Schrödinger Suite* 2012.[34] The grid generation and all the docking runs were performed leaving all the variables at default values in the Glide 5.5[34] program used in single precision docking mode (SP) and saving up to 10 best poses per compound. The obtained results were further submitted to the *Prime MM-GBSA*[34] protocol to estimate the *Free Binding Energy* of the ligands using a minimization shell of 8 Å from the center of mass of the binder.

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Supplementary data

Supplementary data (experimental procedures and characterizations for 2-mercaptobutanoic- and 2-mercaptopentanoic acids. HPLC estimation of the sample purity of 1*H*-pyrazolo[3,4-*e*][1,4]thiazepin-7-ones described in the paper. 3-D Docking images of the enantiomers (4*S*,6*R*)-**9** and (4*R*,6*S*)-**9**. Ligand interaction diagrams of (4*R*,6*S*)-**13**, (4*R*,6*S*)-**14**, (4*R*)-**15**, (4*R*,6*S*)-**16** and (4*R*,6*S*)-**17**) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.04.038.

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