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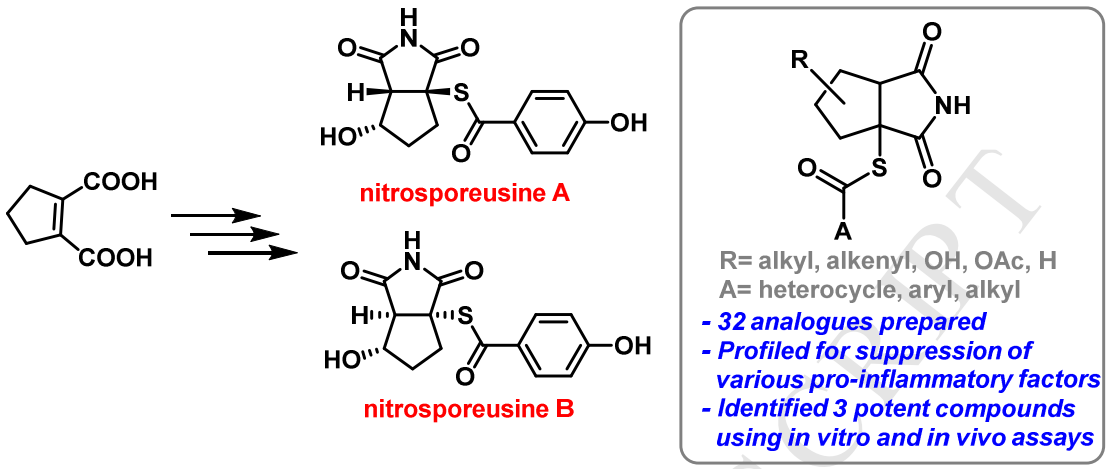
Graphical abstract



Identification of new anti-inflammatory agents based on nitrosporeusine natural products of marine origin

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ABSTRACT

Nitrosporeusines A and B are two recently isolated marine natural products with novel skeleton and exceptional biological profile. Interesting antiviral activity of nitrosporeusines and promising potential in curing various diseases, evident from positive data from various animal models, led us to investigate their anti-inflammatory potential. Accordingly, we planned and synthesized nitrosporeusines A and B in racemic as well as enantiopure forms. The natural product synthesis was followed by preparation of several analogues, and all the synthesized compounds were evaluated for *in vitro* and *in vivo* anti-inflammatory potential. Among them, compounds **25**, **29** and **40** significantly reduced levels of nitric oxide (NO), reactive oxygen species (ROS) and pro-inflammatory cytokines. In addition, these compounds suppressed several pro-inflammatory mediators including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor-κB (NF-κB), and thereby can be emerged as potent anti-inflammatory compounds. Furthermore, all possible isomers of lead compound **25** were synthesized, characterized and profiled in same set of assays and found that one of the enantiomer (-)-**25a** was superior among them.

Keywords. Inflammation, pro-inflammatory factors, nitrosporeusines, maleimycin, *Amano PS* lipase, enzymatic kinetic resolution

1. Introduction

Inflammation is a biological response to harmful stimuli such as pathogens that cause tissue and cell damage.[1] It is considered as a defensive measure taken by the organism to eliminate noxious stimuli and to begin the healing process. It is classified as either acute or chronic, depending on whether it involves a short response or a prolonged one, respectively.[2] During an inflammatory response, mediators, such as pro-inflammatory cytokines (e.g., interleukin-1β (IL-1β), IL-6, IL-12, and the chemokine IL-8), tumor necrosis factors (e.g., TNF-α and TNF-β), interferons (e.g., IFN-γ), eicosanoids (e.g., prostaglandins and leukotrienes) and vasoactive amines (e.g., histamine) are released.[3] The transcription factor nuclear factor-κB (NF-κB), plays a central role in the inflammatory response by regulating the expression of various genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes such as cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS).[4] In spite of the fact that inflammation is primarily a protective response, the chronic and uncontrolled inflammation becomes detrimental to tissues.[5] The inferences of the chronic inflammation in the pathogenesis of arthritis, cancer, cardiovascular, autoimmune as well as viral infections have made it a serious medical issue.[6] Therefore, research has been directed in recent years to develop safer and potent anti-inflammatory drugs to attenuate the severity of inflammation.[6,7] As the human immune system is a complex process involving many factors and can go awry many times, it is very challenging to develop novel efficient chemical entities for treating inflammation.[8]

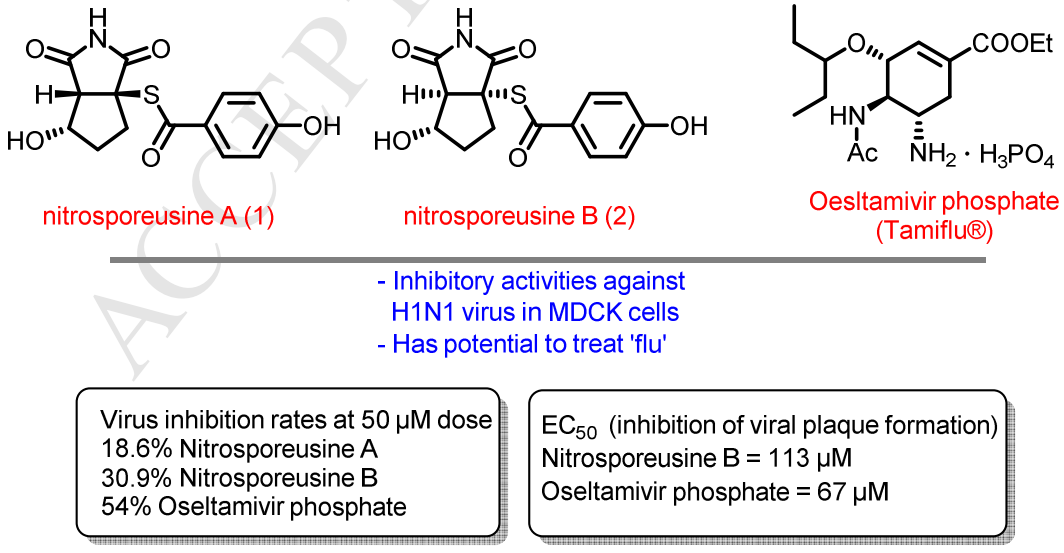


Figure 1: Natural products nitrosporeusines A (**1**) and B (**2**)

As a part of our continuous interest[9] in search of biologically active natural molecules with anti-inflammatory activity, we have come across a novel class of compounds with benzenecarbothioccyclopenta[c]pyrrole-1,3-dione scaffold - nitrosporeusines. Nitrosporeusines A (**1**) and B (**2**) are two new marine natural products having unique skeleton isolated[10] by Lin and co-workers from Arctic Chucki sea (Figure 1). The structures of both compounds were established through detailed NMR and single crystal X ray studies. Biological evaluation of these compounds revealed that both compounds **1** and **2** have promising potential in treating influenza virus strains A/WSN/33(H1N1).[10] Following that, *in vivo* studies by Chen and co-workers showed in mouse models that, nitrosporeusine A has exceptional potential in treating wide range of diseases such as rhinitis, oral ulcer, chronic heart failure, acute renal failure and renal fibrosis and all of them are claimed in a series of patents[11] (see figure 2 for details). These biological activity results reported for nitrosporeusines, in particular with compound **1** are very impressive, and attracted our attention where we planned for a complete study over this novel scaffold. Due to close association of many microbial and viral infections with inflammation,[12] we envisioned to synthesize and study the nitrosporeusines in detail towards its anti-inflammatory potential.

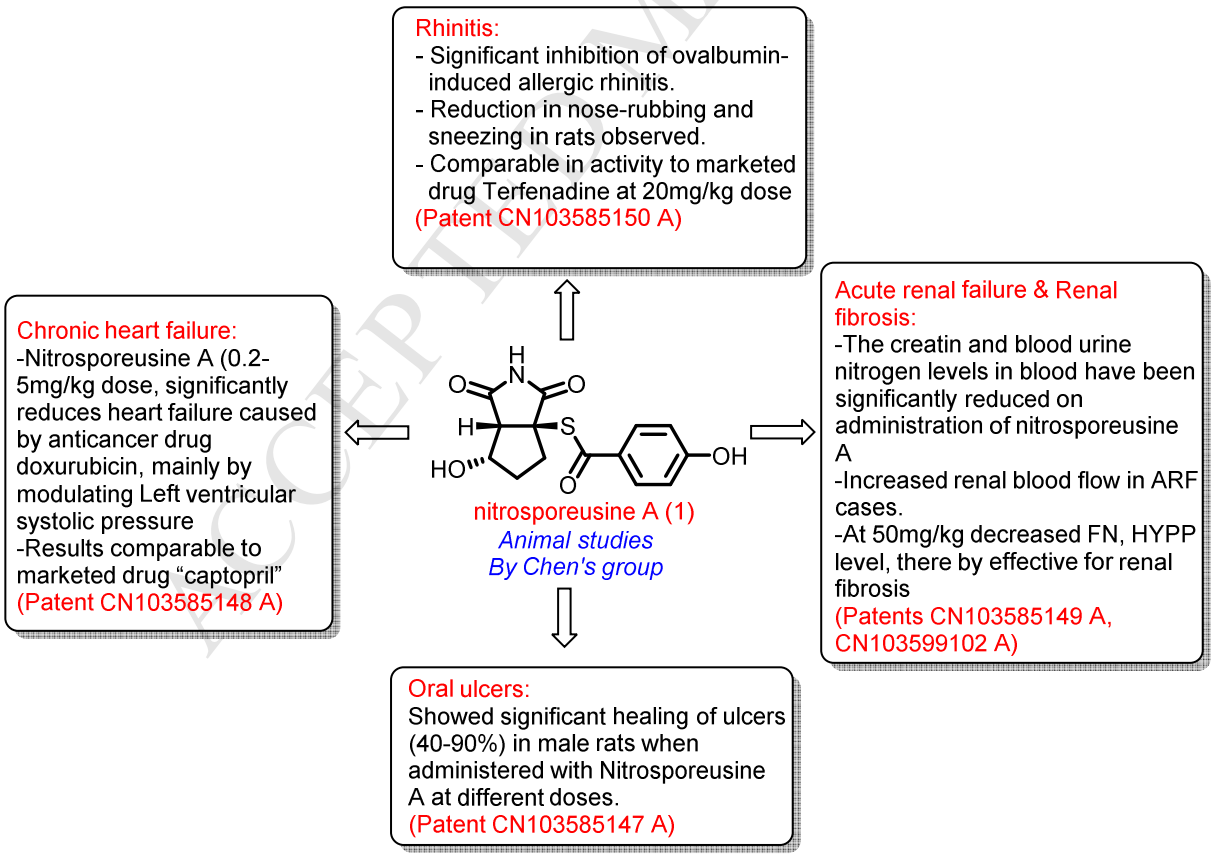


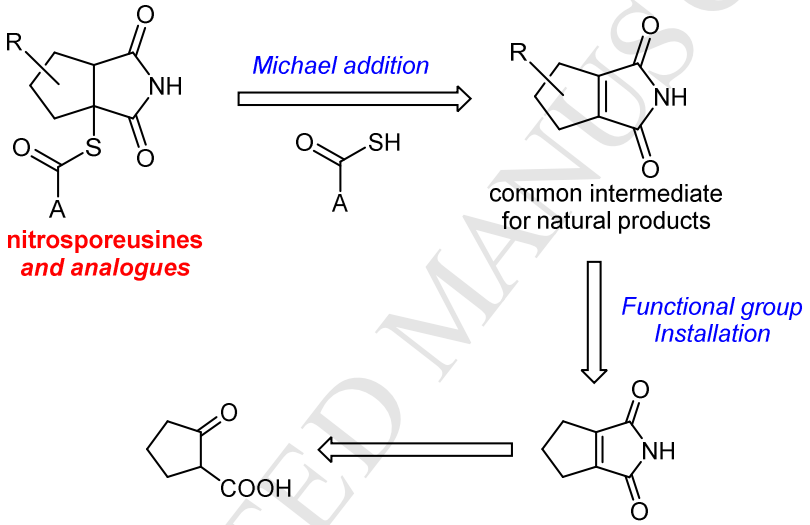
Figure 2: Exceptional activity of nitrosporeusine A (**1**)

Accordingly, the first total synthesis of nitrosporeusines A & B using a simple chemistry was achieved from this group and reported in a preliminary communication.[13] The developed route is flexible and now we extended it to generate a library of analogues around the natural product skeleton. Full account of design, synthesis and biological evaluation on nitrosporeusine scaffold is discussed in the present paper.

2. Results and Discussion:

2.1 Chemistry

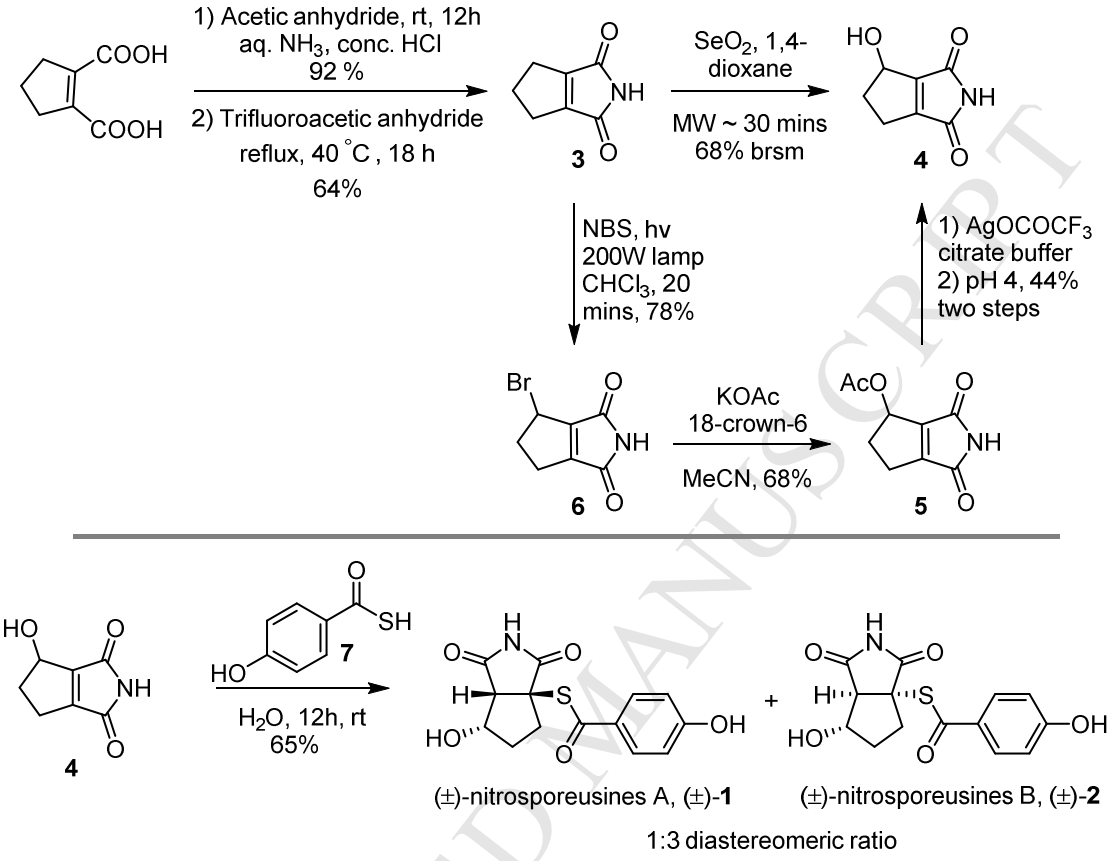
Nitrosporeusines A and B share a common structural core with difference only in relative stereochemistry between hydroxy group and ring junction.



Scheme 1: Approach towards nitrosporeusines and their analogues

So, both the natural products are envisioned to be obtained from a common intermediate – maleimycin, which in turn could be obtained from compound **3**. This maleimide moiety (**3**) could be prepared from cyclopent-1-ene-1,2-dicarboxylic acid in few synthetic operations. While we were planning for the synthesis, we chose a strategy which would be amenable for synthesis of both natural products, as well as analogue synthesis. We planned to exploit the key maleimide intermediate to make several building blocks for analogues. Our initial aim was to prepare compound **3** in good quantities, so following literature procedures,[14a,b] we treated cyclopent-1-ene-1,2-dicarboxylic acid with acetic anhydride/NH_3 which was then subjected to intramolecular condensation using trifluoroacetic anhydride resulting in desired maleimide compound **3** in good yields (Scheme 2). After scaling up the reaction and making sufficient quantities, we first explored the synthesis of nitrosporeusines A and B in racemic forms. Towards that we subjected

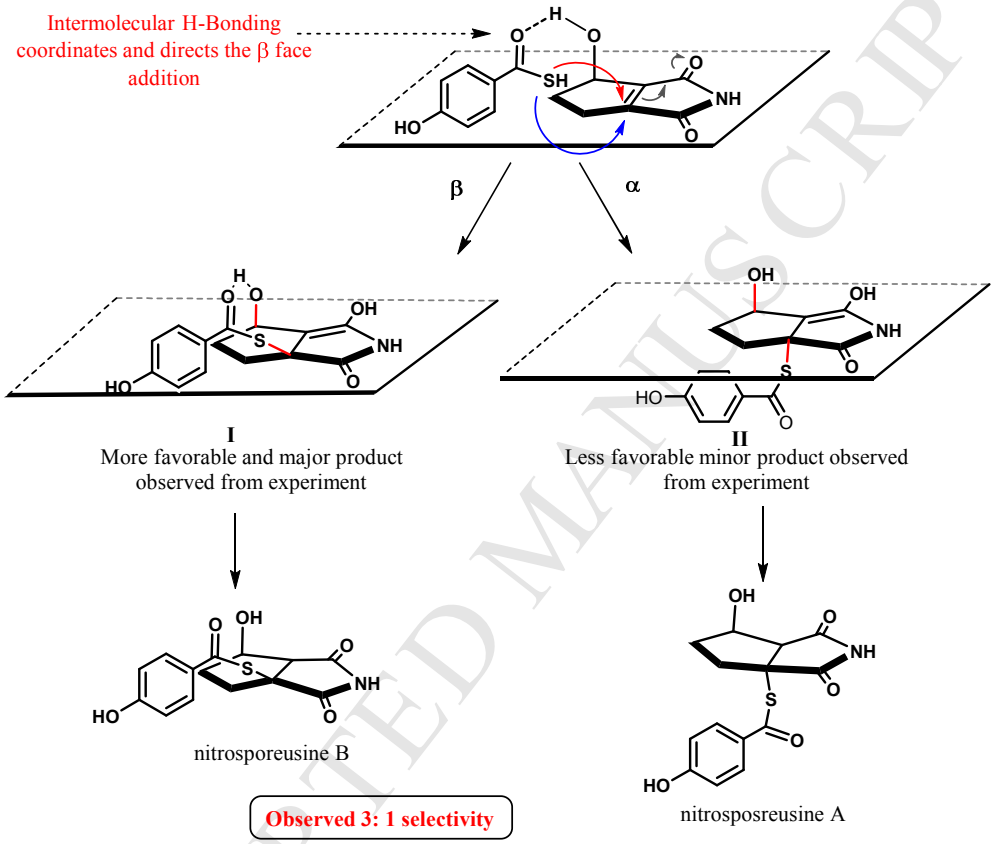
compound **3** to microwave mediated allylic oxidation using selenium dioxide,[15] and after few optimizations we could obtain desired allylic alcohol (**4**) in 68% yield (brsm).



Scheme 2: Synthesis of racemic nitrosporeusines A and B

Alternately, the same alcohol **4** was obtained by allylic bromination (NBS/hυ/$CHCl_3$) followed by acetylation and hydrolysis protocol (Scheme 2). Having the required racemic maleimycin in hand we next prepared required acid fragment for Michael addition. Commercially available 4-hydroxybenzoic acid along with solid Lawesson's reagent was dissolved in acetonitrile and irradiated under microwave conditions (100 ºC/ 15 mins)[16] which gave 4-hydroxybenzothioic *S*-acid (**7**). We then subjected **4** to Michael addition with 4-hydroxybenzothioic *S*-acid (**7**) using water as solvent and obtained both nitrosporeusines A and B in 1:3 diastereomeric ratio (Scheme 2). Both the compounds were racemic, but the diastereomers formed were cleanly separated using silica gel column chromatography, characterized and compared with literature reports.[10] Here, the observed selectivity could possibly be due to strong intermolecular H-bonding between thio-ester carbonyl and hydroxy groups, which directs the incoming nucleophilic addition towards forming intermediate **I** as major compound giving nitrosporeusine B (Scheme 3). The

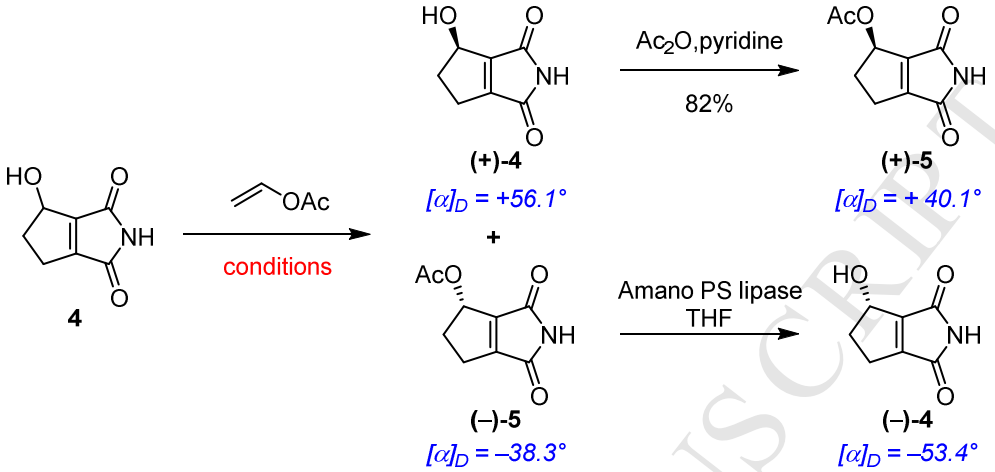
other possibility of addition could result in formation of intermediate **II** resulting in nitrosporeusine A as minor product. Similar H-bonding mediated anti-aza-Michael addition reactions were reported in literature[17] which helped us in proposing this probable mechanism. The successful racemic synthesis of natural products with a simple and efficient route encouraged us to synthesize natural products in enantiopure forms.



Scheme 3: Probable explanation for observed selectivity

The use of enzymatic kinetic resolution as a tool to resolve alcohols is well documented in literature.[18] We subjected alcohol **4** to different conditions in which one of the alcohols would selectively get acetylated and give enantiopure compounds (Scheme 4). Thus after a few attempts with different lipases and conditions (Scheme 4), alcohol **4** was resolved using vinyl acetate in presence of *Amano PS* lipase in dry THF to give acetate (-)-**5** and corresponding pure alcohol (+)-**4** with > 98%ee. The optical purity of (+)-**4** was also confirmed by HPLC and optical rotation values.[19] Here it is interesting to note that (+)-**4** is a natural product maleimycin with good antibacterial and anti-proliferative activities.[14c] We also synthesized (-)-**4** and (+)-**5** from (-

)-**5** and (+)-**4**, respectively, and compared their optical rotation values with enantiomers obtained in enzymatic resolution previously.

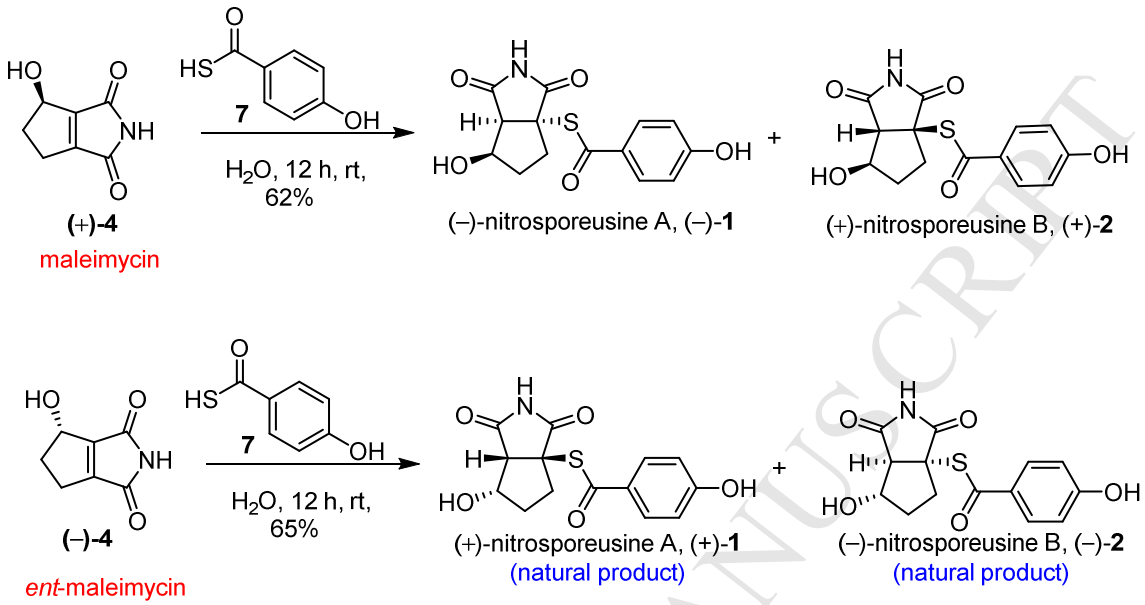


Enzyme	Solvent/time	Observation
Candida antartica lipase B	THF, rt, 24h	10% conversion to (-)-**5**
Candida rugusa lipase	THF, rt, 24h	17% conversion to (-)-**5**
100 wt% Amano PS lipase	THF, rt, 12h	30% yield for isolated (-)-**4**, 87%ee
150 wt% Amano PS lipase	dry THF, rt, 4h	47% yield for isolated (-)-**4,** 99%ee

Scheme 4: Synthesis of enantiopure alcohols

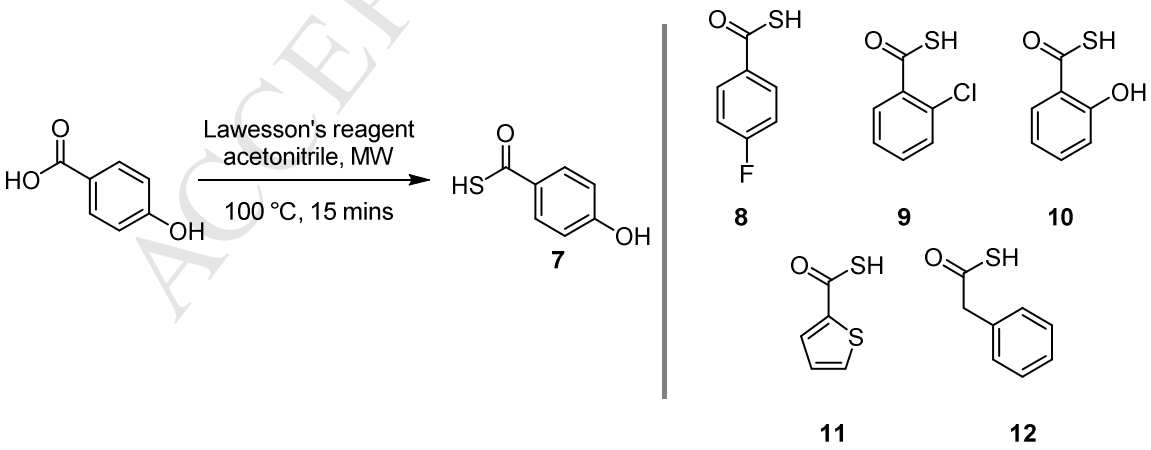
Having (+)-**4** in hand, we then subjected it to Michael addition conditions using 4-hydroxybenzothioic S-acid (**7**) to furnish the target compounds nitrosporeusines A and B in enantiopure forms. To our surprise, the naturally occurring (+)-maleimycin ((+)-**4**) did not yield natural nitrosporeusines (+)-**1** and (-)-**2**, instead their enantiomers (-)-**1** and (+)-**2** were obtained. So, we treated the unnatural maleimycin ((-)-**4**) under same conditions to obtain (+)-nitrosporeusine A and (-)-nitrosporeusine B which are in full agreement with those reported in literature[10] (Scheme 5). Based on above observations, we could probably propose that natural (+)-maleimycin may not be the biogenetic precursor for the natural nitrosporeusines A and B. After documenting all the results obtained,[13] we next planned to expand the scope of these interesting natural products by synthesizing a series of analogues around the nitrosporeusine scaffold and subject them for various anti-inflammatory studies. To obtain close analogues of

nitrosporeusines we exploited the maleimide intermediate (**3**) by synthesizing Michael acceptors **3**, **4**, **5**, **6** around it.

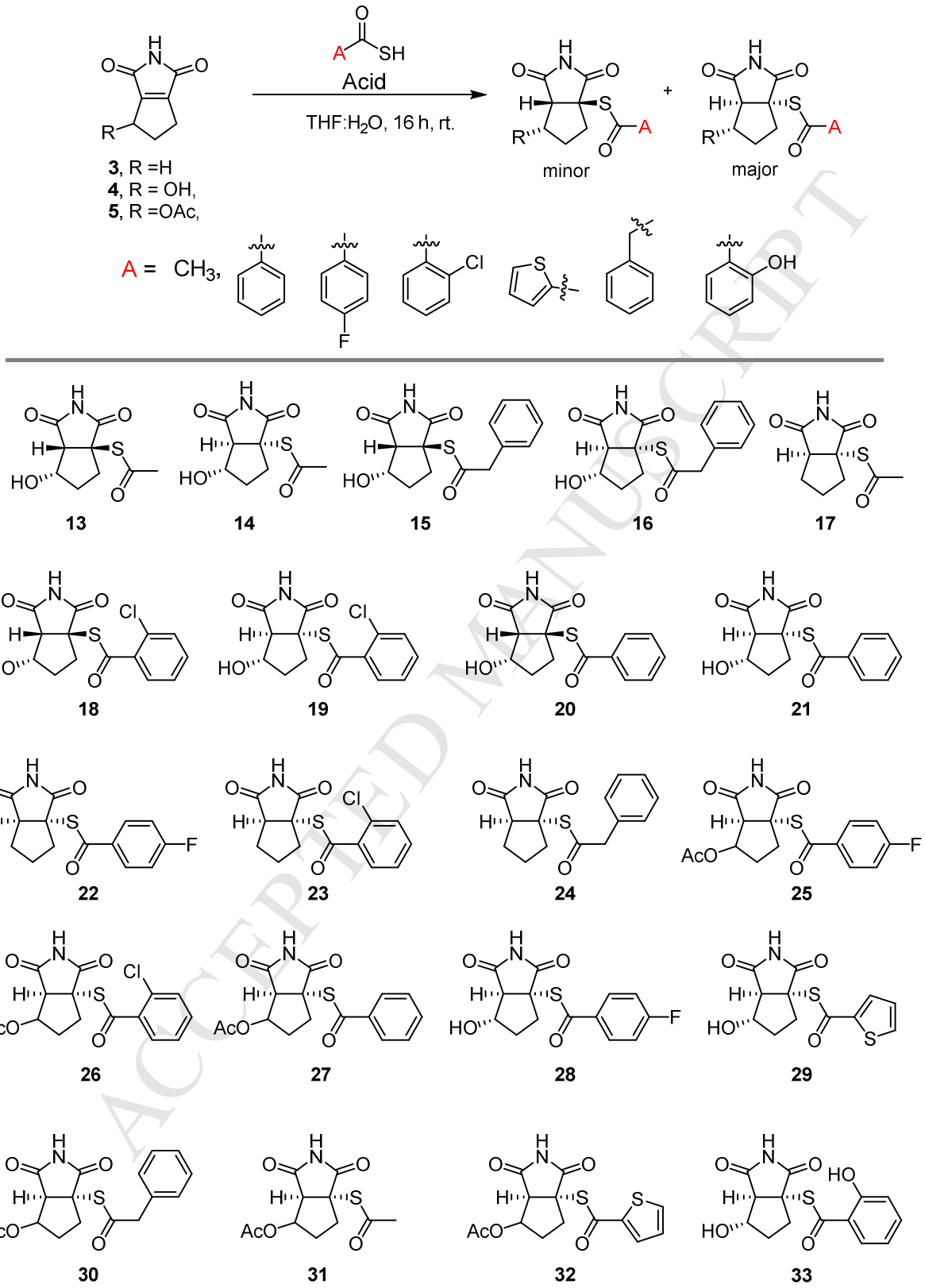


Scheme 5: Synthesis of enantiopure nitrosporeusines

Towards the preparation of thio-acids **7**-**12**, we relied on our earlier method where we used Lawesson's reagent under microwave conditions (Scheme 6) to convert carboxylic acids to corresponding thio-acids.[16] These thio-carboxylic acids are found to dimerise immediately even when stored at 4 °C, so we freshly prepared them when required and partially purified them over short silica column before use.



Scheme 6: Synthesis of thio-acids from corresponding carboxylic acids



Scheme 7: Synthesis of nitrosporeusine analogues

As an initial attempt at synthesis of analogues we subjected **3**, **4** and **5** to Michael addition with thioacetic acid, thiobenzoic acid and various thio-acids **7-12** to get corresponding nitrosporeusine analogues with major being all *syn* isomer (Scheme 7). The diastereomers obtained from **4** were cleanly separated using column chromatography. The stereochemistry of these products was assigned based on the ^{1}H NMR patterns observed in natural products nitrosporeusine A and B. In the ^{1}H NMR spectrum the splitting pattern of the C6 proton which couples with both the adjacent protons at C5 and C7, explains the *syn* or *anti* relation between hydroxy group and the ring junction. In both the natural products the extent of coupling between C6 and C5 protons is more or less the same; but in case of nitrosporeusine A, owing to the *syn* relationship between the C6 and C7 protons, the C6 proton couples with the C7 proton to a larger extent as compared to nitrosporeusine B, where the concerned protons (C6 and C7) are *anti* to each other. As a result, C6 proton of nitrosporeusine A shows a greater extent of splitting than nitrosporeusine B (figure 3). Apart from this, on comparing both natural products, we can also observe that the pattern of C4 – C5 protons is different depending on whether hydroxy group is *syn* or *anti* to ring junction (Figure 3).

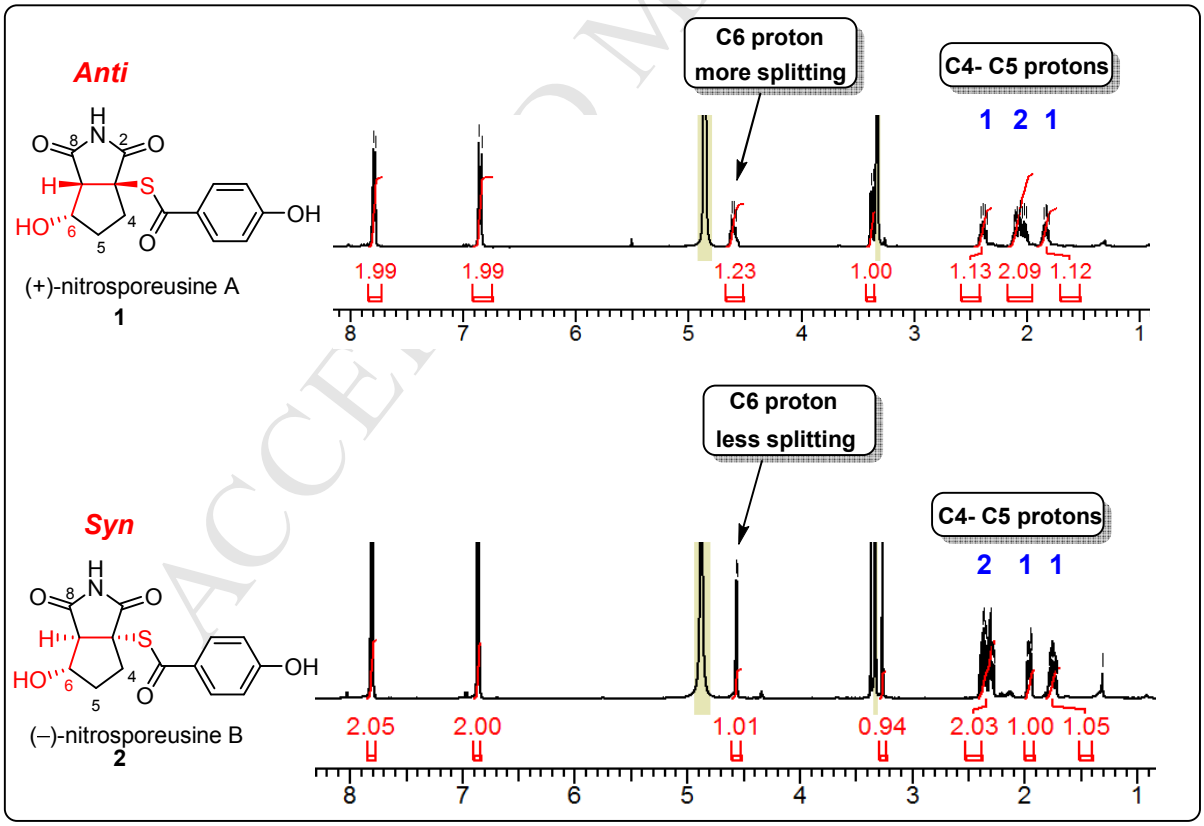
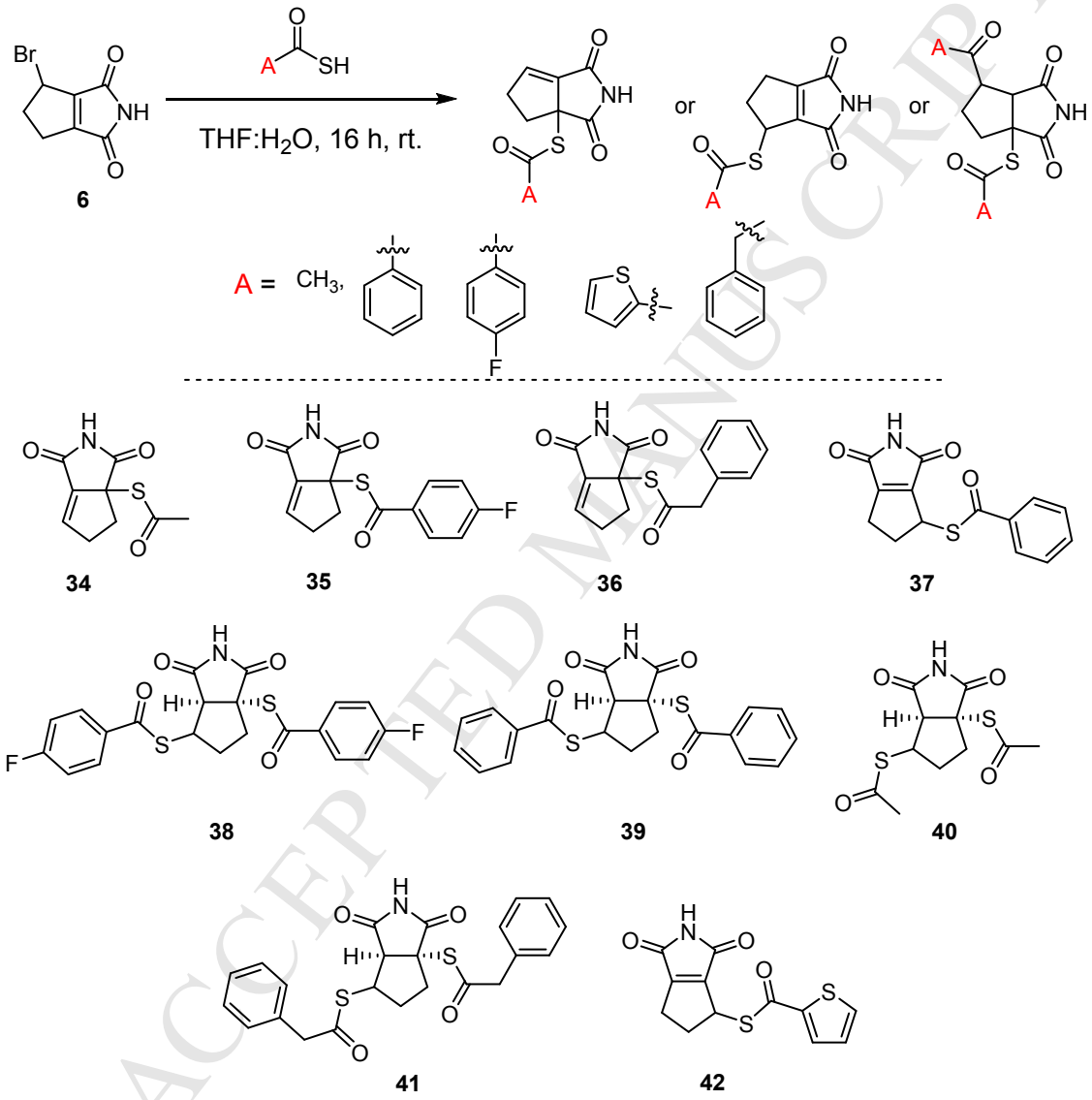


Figure 3: NMR pattern observed for C6, C4, C5 protons in nitrosporeusines

Similar pattern of C6, C4 and C5 protons were observed in ^{1}H NMR spectra of diastereomers obtained from **4** and different thio-acids, which enabled us to assign the relative stereochemistry between hydroxy group and ring junction in them. Here, we synthesized eleven new analogues **13-16**, **18-21** and **28**, **29**, **33** from racemic maleimycin (**4**).



Scheme 8: Synthesis of di-substituted and unsaturated nitrosporeusine analogues

The Michael addition of thioacetic acid, 2-chlorobenzothioic *S*-acid, 2-phenylethanethioic *S*-acid and 4-fluorobenzothioic *S*-acid on compound **3** gave corresponding nitrosporeusine analogues **17** and **22-24**. These compounds were all racemic and are fully characterised by spectral techniques

(^{1}H, ^{13}C, HRMS). Similarly the reaction of different thio-acids on compound **5** gave six new acetate analogues **25**-**27** and **30**-**32** and all compounds are fully characterised. However, in spite of best efforts we could not separate diastereomers formed from **5** but nevertheless diastereomeric acetates **25**-**27**, **30**-**32** we screened as such for biological activity (Scheme 7). In total we made around 21 new analogues using the substrates **3**, **4** and **5**. The synthesis of analogues over compound **6** was then explored where Michael addition using thio-acids gave entirely new and interesting compounds. Instead of simple addition adducts, we observed Michael addition followed by elimination to give olefin analogues which further underwent second Michael addition with thio-acids giving rise to di-substituted analogues. Accordingly, when compound **6** was subjected to Michael addition using 4-fluorobenzothioic *S*-acid, it resulted in compound **35** (Scheme 8). Probably, Michael addition followed by dehydrobromination resulted in compound **35** which is an interesting intermediate for generating a variety of compounds around this skeleton. For example, the newly generated double bond potentially can be used for addition of various nucleophiles, dihydroxylation, allylic oxidation *etc*. Similar olefin analogues were obtained when compound **6** reacts with thioacetic acid and 2-phenylethanethioic *S*-acid. But when the same reaction was performed on **6** using thiobenzoic acid, it resulted in product **37** in good yields, which is the result of substitution of bromine. Compound **37** is also an interesting intermediate towards the library creation. Similar product **42** was obtained when Michael addition performed using thiophene-2-carbothioic *S*-acid. During all these reactions we also isolated compounds **38** – **41** in minor quantities as a mixture of diastereomers which were also considered for biological screening. These compounds have been later prepared exclusively by prolonging the reaction time and addition of more equivalents of respective thio-acids. Following this approach we could prepare compounds **34** – **37** and **40** – **42**. In total by using different Michael acceptors and thio-acids we prepared 32 new compounds which were well characterized by spectral techniques. The inflammatory studies on all compounds were planned in a systematic manner where first cytotoxicity and NO inhibition studies were to be carried out. Based on the results we thought to select few compounds which would be analysed for their inflammatory potential with different pro-inflammatory factors.

2.2 Biology

2.2.1 Anti-inflammatory activity

First, all compounds were evaluated for their inhibition of NO production in LPS stimulated RAW264.7 cells and nitrite levels, a strong metabolite of NO, were measured in culture media using Griess reagent.[20] During inflammation, large amount of NO are produced and in turn amplify inflammatory response to multiple fold. The primary results indicated that almost all compounds evidently decreased NO level in LPS treated cell as shown in Table 1. Simultaneously, cytotoxicity of all compounds were assessed using MTT assay and IC_{50} was calculated (Table 1).

Table 1: Cytotoxicity and LPS induced NO inhibitory activity in RAW 264.7 cells

	Cytotoxicity (A)		NO inhibition (B)		
Com-pound	IC_{50} (µM)	95% confidence interval (µM)	IC_{50} (µM)	95% confidence interval (µM)	Selectivity index (A/B)
(-)-1	616.1	513.7-802.9	42	31.2-56.3	14.66905
(+)-1	1015.6	856-1281.4	71.8	60-84.6	14.14485
(±)-2	433.8	307.4-592.6	225.3	182.8-289.4	1.925433
(+)-2	2914.9	2307-3449.4	126.8	98.3-146.7	22.98817
(-)-2	1383.9	1045.3-1536.8	140.5	118.1-157.3	9.849822
(-)-4	216.7	103.8-354.7	95.9	79.4-116.3	2.259645
(+)-4	319.2	284.3-367.8	121.3	96.3-152.8	2.631492168
(-)-5	128	98.4-137.7	866.9	682.3-993.5	0.147653
13	558.5	472.6-637.2	147.9	112.5-186.9	3.7762
14	856.7	622.4-983.3	832.8	664.7-1004.7	1.028698
15	834.5	727.4-967.9	132	107.7-165.2	6.32197

16	773.7	682.8-867.8	142.8	116.4-204.7	5.418067
17	674.4	493.7-819.6	59.2	46.8-76.9	11.39189
18	667.7	512.3-788.6	127.3	106.3-146.8	5.24509
19	717.1	604.6-833.6	178.3	119.7-213.4	4.021873
20	479.4	294.5-601	72.4	49.8-87.4	6.621547
21	17063	15948-18666	65.2	47.8-78.1	261.7025
22	383.6	305.1-459.8	389.8	327.5-466.1	0.984094
23	834.5	773.8-923.1	59.8	43.6-77.5	13.95485
24	425.6	301.4-556.2	56.9	33.5-83.6	7.479789
25	**12135.6**	**11853-12738**	**34.3**	**24-43.2**	**353.8076**
26	9076.1	8113-10263	101.4	74.1-121.8	89.50789
27	1912	1628.4-2249.9	64.3	52.9-93.8	29.73561
28	473.7	355.6-513.9	318	271.2-380	1.489623
29	**10872.1**	**9332.1-11382**	**36.6**	**24.5-46.1**	**297.0519**
30	1607.3	1283.5-1983.5	68.2	44.7-87.6	23.56745
34	184.5	109.2-228.5	20.5	15.6-26.1	9
36	93.07	84.7-109.3	30.6	18.9-43.7	3.041503
38	210.6	183.2-237.4	98.3	83.6-123.6	2.142421
40	**10007.6**	**9438.3-10837.8**	**19.8**	**14.5-25.4**	**505.4343**
41	710.8	525.6-984.4	57.5	42.8-76.1	12.36174
42	26.7	13.5-42.6	65	48.6-74.8	0.410769

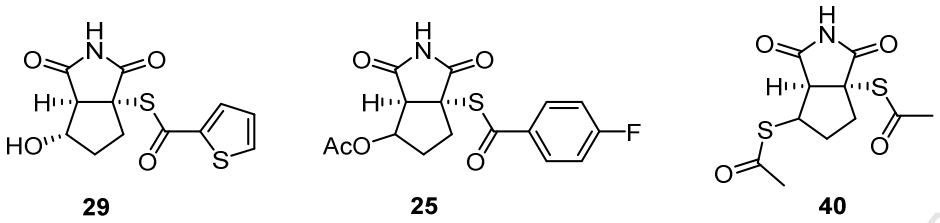


Figure 4: Selected compounds based on cytotoxicity and no inhibition potential

However, the compounds showing effective NO inhibitory activity with low cytotoxicity were screened based on the selectivity index (cytotoxicity IC_{50}/NO inhibition IC_{50}). Compounds **25**, **29** and **40** were selected for further study (figure 4), since they exhibited superior selectivity indices (≥300) among all the derivatives. Further, the anti-inflammatory potency of compounds **25**, **29** and **40** were determined by measuring the level of intracellular reactive oxygen species (ROS) in LPS treated RAW 264.7 cells. ROS generation is a key marker for inflammation and is reported in several cases as a triggering factor for apoptosis.[21]

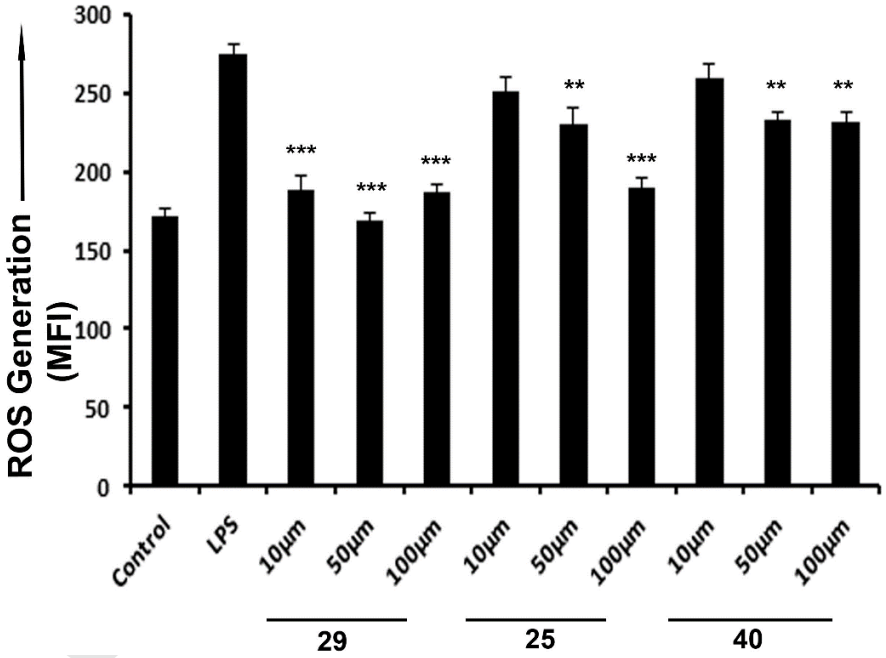


Figure 5: Effects of compounds on ROS level in LPS stimulated RAW 264.7 cells. The plot represents the mean fluorescence intensity (MFI) of ROS generation in presence of three selected compounds. Data represent mean ± SD of three independent experiments. **p<0.01 and ***p<0.001in comparison to LPS-treated values.

As depicted in Figure 5, increase of ROS in LPS treatment was reduced significantly in presence of all these three compounds in a dose dependent manner as analysed by mean fluorescence intensity (MFI). Inflammation is the body's first response of the immune system to infection or irritation. During the inflammatory process, large quantities of the inflammatory mediators are produced by the inducible isoforms of iNOS and COX-2.[22] Thus we checked the levels of iNOS and COX-2 by immunoblotting in response to drugs treatment in LPS administered cells. We found that 50 μM of all three selected compounds are efficient in reducing iNOS and COX-2 levels compared to LPS (Figure 6A & 6B).

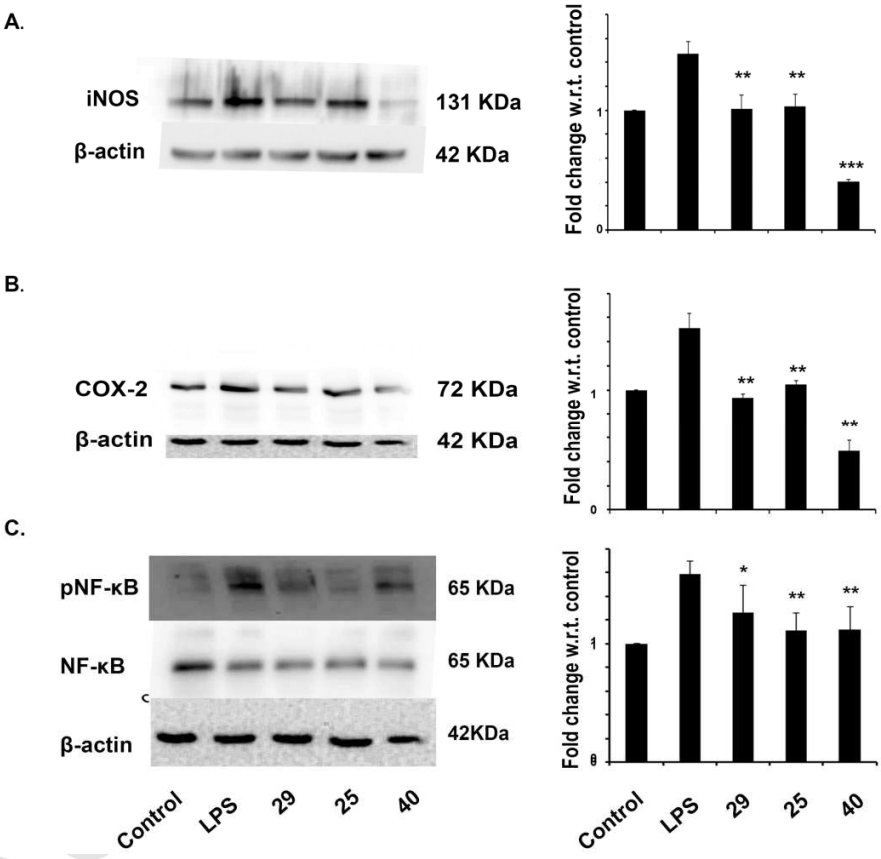


Figure 6: Protein isolated from RAW 264.7 cells of control, LPS and LPS+drug conditions were analysed by immunoblot. The graphs represent the densitometric quantification of protein bands-iNOS (A), COX-2 (B) and phospho-NFκB (C) normalised to β-actin. Values represent mean ± SD from 3 independent experiments. *p<0.05, **p<0.01 and ***p<0.001 in comparison to LPS treated values.

Since NF-κB activation is an important marker of LPS-induced inflammation, we assessed influence of these compounds on NF-κB activation. NF-κB is present in the cytoplasm as an inactivated dimer composed of p65 and p50 subunits. In response to inflammatory stimuli, IκB is phosphorylated and degraded, and NF-κB is released and translocated into the nucleus,[23] where it binds promoter region of many inflammatory genes, including iNOS, COX-2, and TNF-α and augments their expression.[24] Our results showed that **25**, **29** and **40** potentially decreased LPS induced NF-κB activation as analysed phosphorylated NF-κB level (pNF-κB) by western blot (Figure 6C).

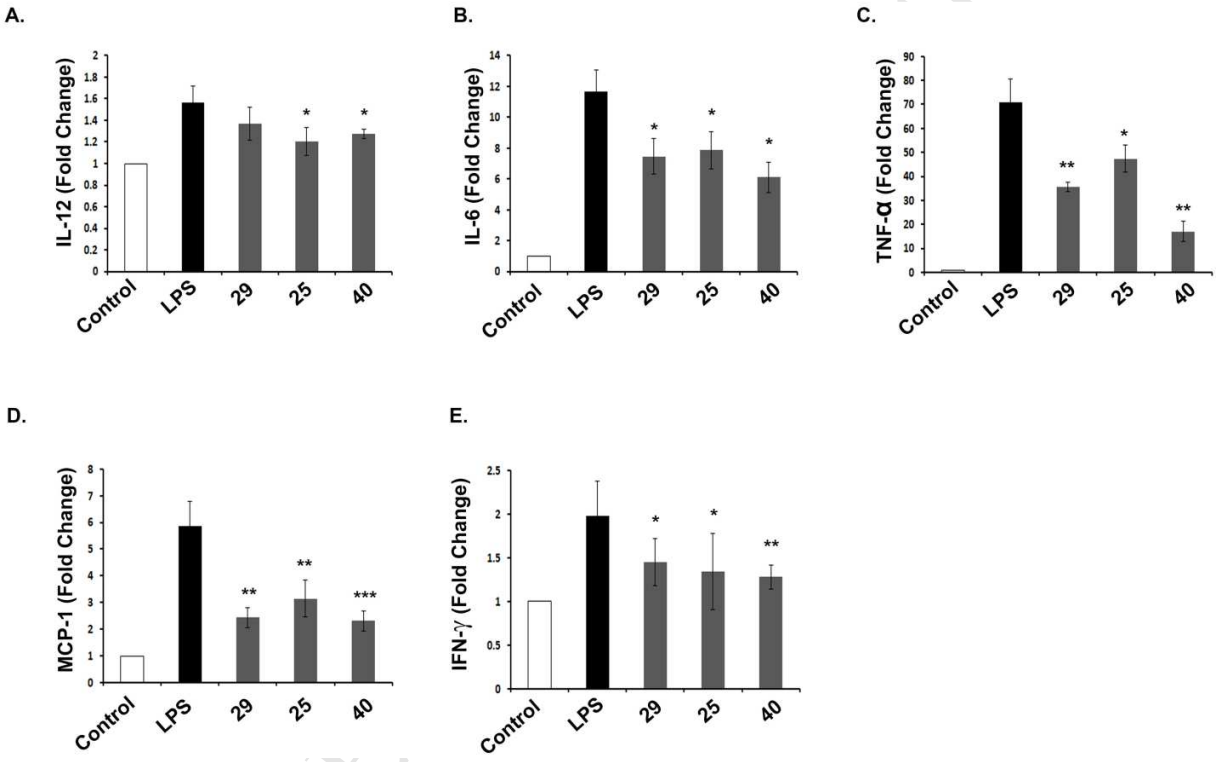


Figure 7: Cytokine Bead assay (CBA) analysis of protein extract isolated from RAW 264.7 cells treated with LPS along with compounds. Addition of compounds (50 µM) revealed substantial decrease in the levels of IL-12 (A), IL-6 (B), TNF-α(C),MCP-1 (D) and IFN-γ (E) compared to LPS treated cells. Data represent mean ± SD of three independent experiments. *p<0.05,**p<0.01 and ***p<0.001in comparison to LPS treated values.

Under the pathogenic attack in our body, activated macrophages release numerous pro-inflammatory cytokine and inflammatory mediators.[25] Hence, the macrophage cell line provides an excellent model for drug screening and evaluation of potential inhibitors of the inflammatory

response. LPS treatment can induce inflammation, resulting in the extreme production of numerous pro-inflammatory mediators including IL-12, TNF-α, IFN-γ, MCP-1 and IL-6. However, the anti-inflammatory activity of compounds **25**, **29** and **40** were evaluated by measuring the expression of these inflammatory cytokines in LPS and compound treated RAW264.7 cells by CBA. As evidenced from figure 7, it was found that 50 µM dose of all these compounds effectively suppressed pro-inflammatory mediators.

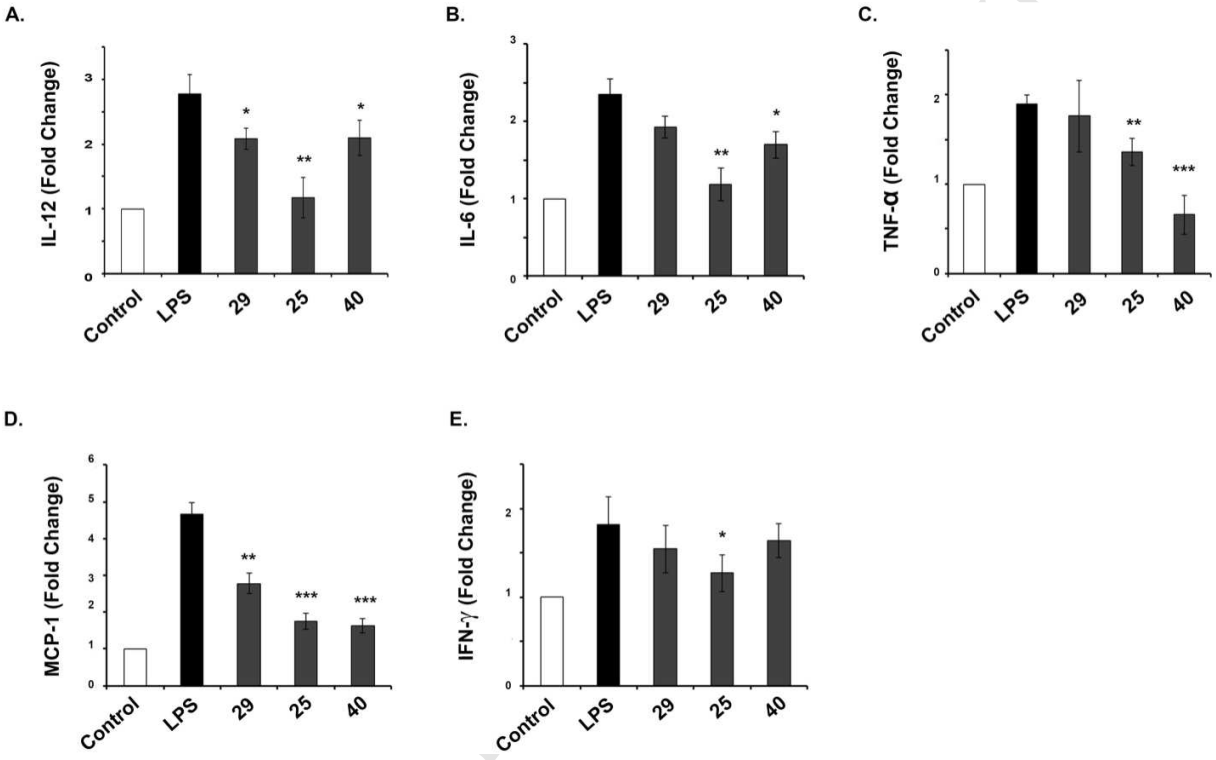
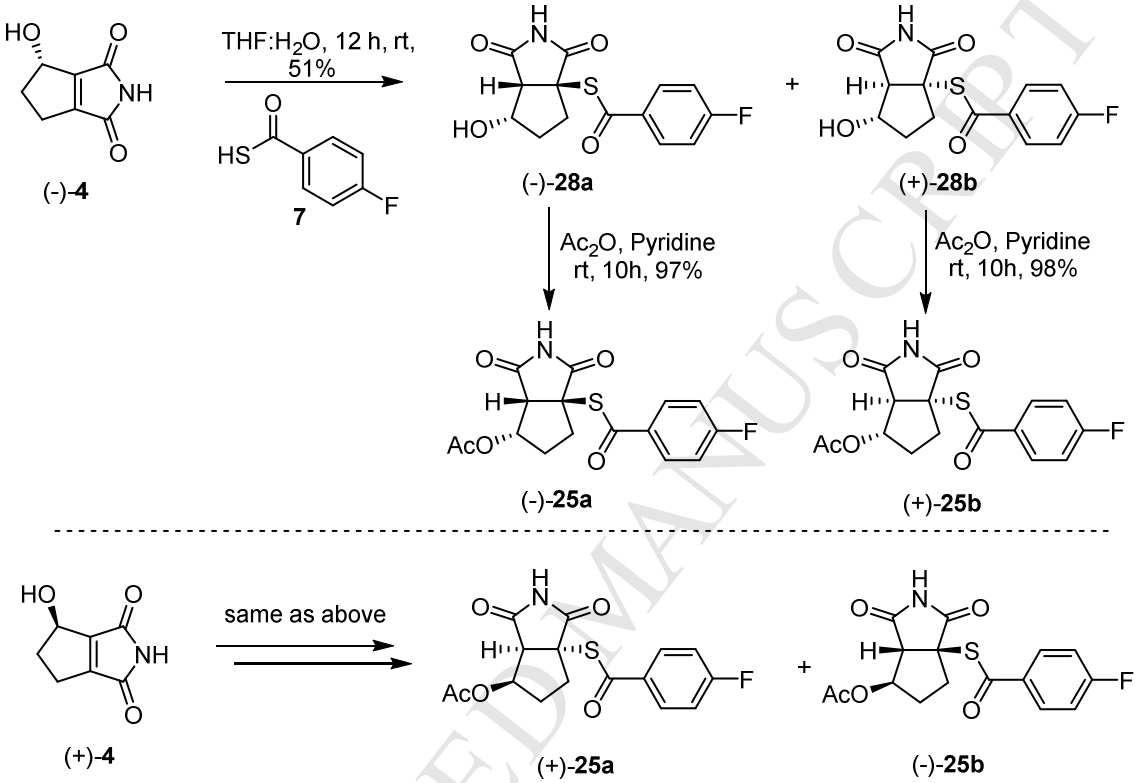


Figure 8: Mouse serum samples were used to study anti-inflammatory activity of drugs *in vivo*. There were significant reductions in the levels of IL-12 (A), IL-6 (B), TNF-α (C), MCP-1 (D), and IFN-γ (E) in compounds (10 mg/kg body weight) treated serum samples compared to only LPS injected mice. Values represent mean ± SD of serum samples collected from five mice. *p<0.05, **p<0.01 and ***p<0.001 in comparison to LPS treated values.

In vivo testing is often employed over *in vitro* because it is better suited for observing the overall effects of an experiment on a living subject. Therefore, we next investigated anti-inflammatory activity of compounds *in vivo* using serum samples collected intracardially from mouse. We measured levels of pro-inflammatory cytokines by CBA and based on the results, compound **25**

was found very promising in *in vivo* though other two compounds were also effective in reducing cytokines level when compared with LPS treated group (Figure 8). As evident from all *in vitro* and *in vivo* studies, compound **25** showed the most potent anti-inflammatory activity among them.



Scheme 9: Synthesis of active enantiopure analogues of nitrosporeusines

So, we planned to prepare four optically pure isomers of **25** to further understand whether the spatial arrangements contribute to anti-inflammatory activity. Towards this we relied on enantiopure compounds (-)-**4** and (+)-**4** to obtain desired alcohols which were then converted to respective enantiopure acetates. Compound (-)-**4,** on subjection to previously optimized Michael addition conditions with 4-fluorobenzothioic *S*-acid gave desired adducts (-)-**28a** and (+)-**28b** in approximately 1:5 diastereomeric ratio. Both the compounds were then carefully separated using silica gel chromatography and characterized. The compound (+)-**28b** was then acetylated using Ac_2O/pyridine conditions to obtain enantiopure acetate (+)-**25b**. Similarly, (-)-**28a** was converted to acetate (-)-**25a** (Scheme 9). Using similar reactions with (+)-**4** as starting material, acetate compounds (+)-**25a** and (-)-**25b** have been obtained in enantiopure forms.

*2.2.2. Anti-inflammatory activity of enantiomers of compound **25***

With all the four diastereomers of **25** in hand, we again assessed them through *in vitro* and *in vivo* assays to identify the potent lead compound among them.

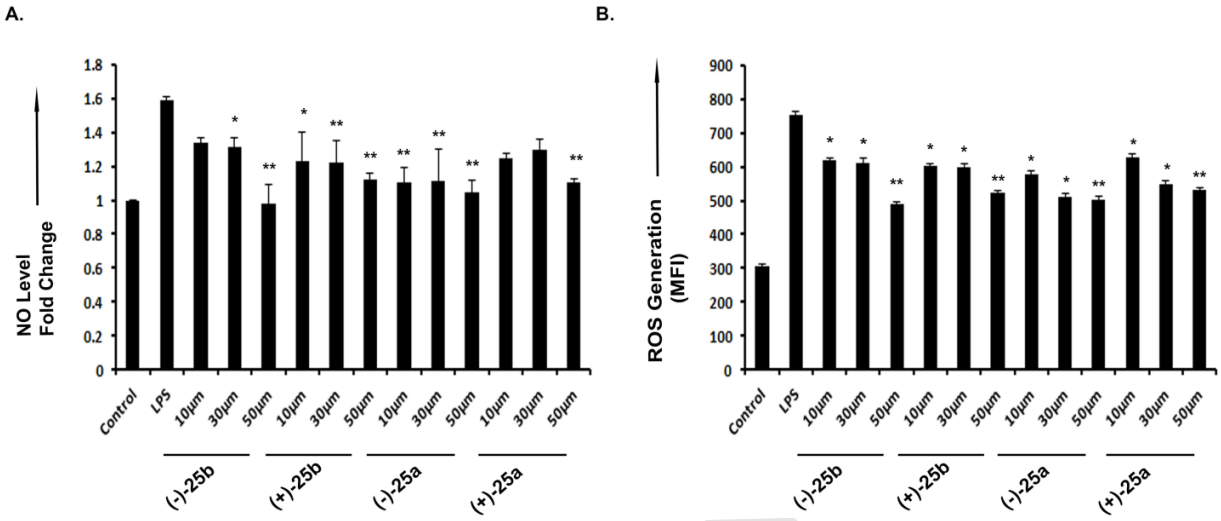


Figure 9: Effects of enantiomers on NO and ROS level in LPS stimulated RAW 264.7 cells. The plot represents the fold change of NO (A) and mean fluorescence intensity (MFI) of ROS generation (B) in presence of four enantiomers. Data represent mean ± SD of three independent experiments. *p<0.05 and **p<0.01 in comparison to LPS-treated values.

The anti-inflammatory efficiency of enantiomers was determined by measuring the NO level in culture supernatants as well as intracellular ROS in LPS treated RAW 264.7 cell. Although marked reduction in NO and ROS level were observed in all enantiomers treated cells, (-)-**25a** showed most potential inhibition among them (Figure 9). Next, we checked the levels of iNOS, COX-2 and pNF-κB by immunoblotting in response to enantiomers treatment in LPS treated cells. Among them, enantiomer (-)-**25a** most significantly attenuated NF-κB activation and a comparable reductions in iNOS and COX-2 expression were observed (Figure 10).

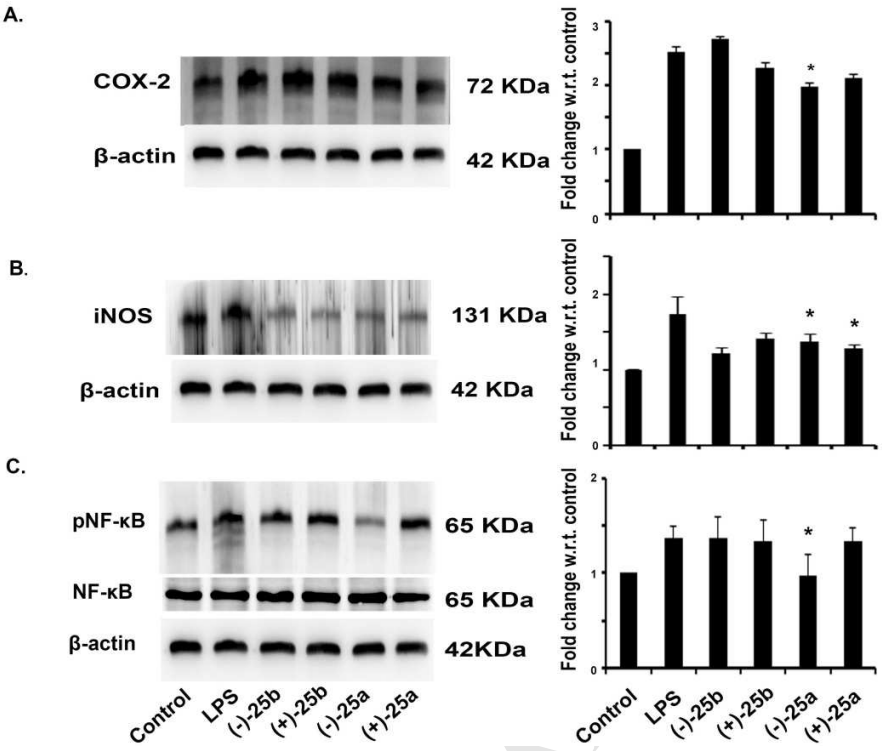


Figure 10: Protein isolated from RAW 264.7 cells of control, LPS and LPS + enantiomer (30 μM) conditions were analysed by immunoblot. The graphs represent the densitometric quantification of protein bands- COX-2 (A), iNOS (B) and phospho-NF-κB (C) normalised to β-actin. Values represent mean ± SD from 3 independent experiments. *p<0.05 in comparison to LPS treated values.

Consistently, enantiomer (-)-**25a**, in comparison to others, significantly reduced the expressions of pro-inflammatory cytokines in LPS treated cells as assessed through CBA analysis (Figure 11). From all the above biological studies it may be concluded that compound (-)-**25a** showed potent anti-inflammatory activity among all the analogues and its structure and stereochemistry closely relates to nitrosporeusine A. Incidentally, nitrosporeusine A showed significant activity in various animal models for treatment of different diseases (as shown in figure 2).

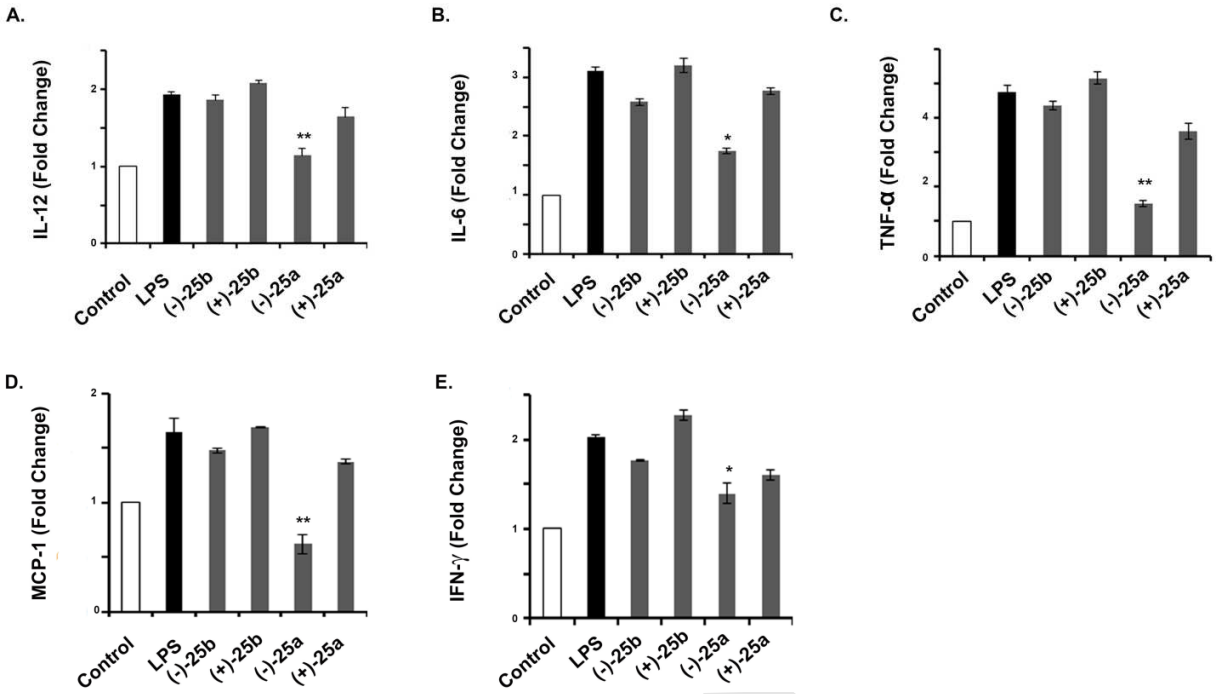


Figure 11: CBA analysis of protein extract isolated from RAW 264.7 cells treated with LPS along with enantiomers. Addition of compounds (30 µM) showed substantial decrease in the levels of IL-12 (A), IL-6 (B), TNF-α (C),MCP-1 (D) and IFN-γ (E) compared to LPS treated cells. Data represent mean ± SD of three independent experiments. *p<0.05,**p<0.01 in comparison to LPS treated values.

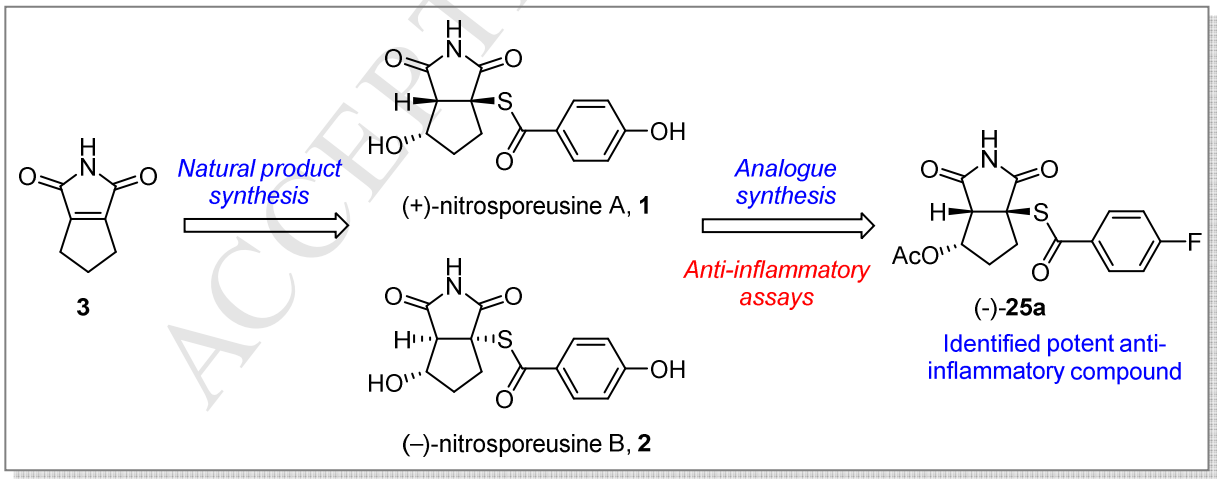


Figure 12: Identification of active nitrosporeusine analogue through synthesis

3. Conclusion

The intriguing structure and exceptional biological activity reported for nitrosporeusines A and B has encouraged us to plan and execute a medicinal chemistry program around nitrosporeusine scaffold in identifying new anti-inflammatory agents. We achieved first synthesis of nitrosporeusines A and B in both racemic and enantiopure forms using short and simple route with key steps involving allylic oxidation (SeO_2) and Michael addition (in water). We synthesized four isomers of the natural products in enantiopure forms by employing gram scale kinetic enzymatic resolution method using *Amano PS* lipase. Later, we diverted our efforts towards synthesis of 32 close analogues of nitrosporeusines. All the synthesized compounds were profiled for their anti-inflammatory potential in different assays. The initial screening with respect to NO inhibition and cytotoxicity has shown that three of the compounds are promising which prompted us to profile them in further studies. Accordingly, compounds **25, 29** and **40** have been studied in detail with various inflammatory markers and **25** showed the most promising anti-inflammatory potential. Four enantiomers of compound **25** were further prepared and evaluated for their anti-inflammatory activity using same assays, and finally the enantiomer (-)-**25a** was recognized as the potential lead from this project.

4. Experimental Section

4.1 Chemistry

All reactions were carried out in oven-dried glassware under a positive pressure of argon or nitrogen unless otherwise mentioned with magnetic stirring. Air sensitive reagents and solutions were transferred *via* syringe or cannula and were introduced to the apparatus *via* rubber septa. All reagents, starting materials and solvents were obtained from commercial suppliers and used as such without further purification. Reactions were monitored by thin layer chromatography (TLC) with 0.25 mm pre-coated silica gel plates (60 F254). Visualization was accomplished with either UV light, or by immersion in ethanolic solution of phosphomolybdic acid (PMA), *para*-anisaldehyde, 2,4-DNP, $KMnO_4$, Ninhydrin solution or Iodine adsorbed on silica gel followed by heating with a heat gun for ~15 sec. Column chromatography was performed on silica gel (100-200 or 230-400 mesh size). Deuterated solvents for NMR spectroscopic analyses were used as received. All ^{1}H NMR and ^{13}C NMR spectra were obtained using a 200 MHz, 400 MHz, and 500 MHz spectrometer and coupling constants were measured in Hertz. In some of the compounds,

we observed doubling of peaks due to diastereomers. All chemical shifts were quoted in ppm relative to TMS, using the residual solvent peak as a reference standard. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. HRMS (ESI) were recorded on ORBITRAP mass analyzer (Thermo Scientific, Q Exactive). Mass spectra were measured with ESI ionization in MSQ LCMS mass spectrometer. Infrared (IR) spectra were recorded on a FT-IR spectrometer. Chemical nomenclature was generated using Chembiodraw ultra 14.0. Melting points of solids were measured in melting point apparatus. Optical rotation values were recorded on P-2000 polarimeter at 589 nm.

Experimental procedures for the preparation of compounds **1**, **2**, **3**, **4**, **5**, **6** as well as HPLC copies of **4**, (-)-**4**, (+)-**4** has been reported previously by us.[18] Data pertaining to the characterization and purity of each of the compounds (-)-**1**, (+)-**1**, (+)-**2**, (-)-**2**, **3**, **4**, (-)-**4**, (+)-**4**, **5**, **6** have also been given earlier.[18] Here all the synthesized analogues were tested for purity on C18 column and found to be greater than 90% pure. Compounds **35**, **37**, **39** were not considered for screening due to lack of HPLC purity.

4.1.1. Procedure A: General procedure for synthesis of analogues through Michael addition

In a round-bottomed flask equipped with a magnetic stirrer, thioacid (1.1 eq.), unsaturated compound (Michael acceptor) (1 eq.), and THF:water (1:1) were charged. The reaction mixture was stirred vigorously at room temperature for 10 to 12h, then it was diluted with EtOAc and extracted twice (2x3 mL). The combined organic layer was washed with saturated aqueous $NaHCO_3$ solution followed by brine solution and concentrated *in vacuo* to obtain a crude mixture which was purified by column chromatography (silica gel; EtOAc/Petroleum ether) to obtain the desired nitrosporeusine analogues.

#All the products are isolated in 55-71% overall yield.

4.1.2. Procedure B: General procedure for synthesis of thio-acids

To a solution of substituted aromatic acid (1 eq.) in dry acetonitrile was added Lawesson's reagent (0.5 eq.) and was subjected to microwave irradiation in closed vessel at 100 °C for 15 minutes (Antonpaar monowave 300 instrument). The reaction mixture was evaporated to dryness and the crude residue obtained was diluted with EtOAc and washed several times with 1N HCl,

then with brine solution and dried over anhydrous Na_2SO_4. The combined organic layers were concentrated *in vacuo* and purified by silica gel column chromatography with elution of 20-30% EtOAc/Petroleum ether.

* All thio-acids are prepared freshly before reaction and used immediately for reactions

4.1.3. Procedure C: General procedure for acetylation

To a solution of alcohol (1 eq.) in pyridine as solvent was added 1.5 eq. of acetic anhydride and 0.1 eq. of dimethylaminopyridine (DMAP) and stirred at room temperature for 6-8 hours. The reaction was diluted with ethylacetate and slowly added with 1N HCl until pH turns acidic. The layers were separated and organic layer was washed with water, brine and dried over anhydrous Na_2SO_4. The crude reaction reaction mixture obtained upon concentrating in vacuo was purified over column chromatography (silica gel; 5-10% EtOAc/CH_2Cl_2) to obtain the desired acetate compounds.

4.1.4. S-((3aS,6S*,6aR*)-6-Hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) ethanethioate (13) and S-((3aR*,6S*,6aS*)-6-Hydroxy-1,3-dioxohexahydrocyclopenta [c]pyrrol-3a(1H)-yl) ethanethioate* **(14)**

Following general procedure A alcohol **4** has been treated with thioacetic acid to get diastereomers **13** and **14** in approximately 1:1.2 ratio. Compound **13** (20 mg) obtained as white solid. IRυ_{max} (film) 3809, 1708, 1692, 1515 cm^{-1}; ^1H NMR (400 MHz, CD_3OD) δ 4.60-4.56 (m, 1H, $CH_2C\mathbf{H}OH$), 3.34-3.30 (d, 1H, (OH)CHC**H**CONH), 2.33 (s, 3H, C**H**$_3$CO), 2.31-2.28 (m, 1H, (OH)CHCH_2C**H**$_2$C(SCOCH_3)) 2.02-1.89 (m, 1H, (OH)CHCH_2C**H**$_2$C(SCOCH_3)), 1.88-1.81 (m, 1H, (OH)CHC**H**$_2$CH_2C(SCOCH_3)), 1.79-1.77 (m, 1H, (OH)CHC**H**$_2$CH_2C(SCOCH_3)); ^{13}C NMR (100 MHz, CD_3OD) δ 198.0 (S-**C**=O), 181.0 (SC**C**ONHCOCH), 177.1 (SCCONH**C**OCH), 73.9 (CH_2**C**HOH), 61.0 ((HO)CH**C**HCONH), 60.7 (CH_3COS**C**CH), 35.2, 33.4, 29.8 (**C**H_3CO); HRMS (ESI) *m/z* calculated for $C_9H_{11}NO_4S$ $[M+Na]^+$ 252.0301, found 252.0299. RT_{HPLC} 5.538 min, purity >90%, 25:75 H_2O/ MeOH. Compound **14** (23 mg) obtained as white solid. Melting Point 120-129 °C; IRυ_{max} (film) 3809, 1708, 1692, 1515 cm^{-1}; ^1H NMR (400 MHz, CD_3OD): δ 4.53-4.51 (m, 1H, $CH_2C\mathbf{H}OH$), 3.17-3.15 (d, 1H, (OH)CHC**H**CONH), 2.31 (s, 3H, C**H**$_3$CO), 2.23-2.19 (m, 2H, $CH_2C\mathbf{H}_2C(SCOCH_3)$), 1.89-1.88 (m, 1H, (OH)CHC**H**$_2$CH_2C(SCOCH_3)),

1.70-1.65 (m, 1H, (OH)CHC**H**$_2$CH$_2$C(SCOCH$_3$)); ^{13}C NMR (100 MHz, CD$_3$OD): δ 196.9 (S-**C**=O), 179.6 (SC**C**ONHCOCH), 176.7 (SCCONH**C**OCH), 74.8 (CH$_2$**C**HOH), 63.6 ((HO)CH**C**HCONH), 58.3 (CH$_3$COS**C**CH), 32.9, 31.7, 28.0 (**C**H$_3$CO); HRMS (ESI): *m/z* calculated for $C_9H_{11}NO_4S$ $[M+Na]^+$ 252.0301, found 252.0299. RT_{HPLC} 4.22 min, purity >95%, 10:90 H$_2$O/ MeOH.

4.1.5. S-((3aS,6S*,6aR*)-6-Hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 2-phenylethanethioate* ***(15)*** *&* *S-((3aR*,6S*,6aS*)-6-Hydroxy-1,3-dioxohexahydrocyclo penta[c]pyrrol-3a(1H)-yl) 2-phenylethanethioate* **(16)**

Following general procedure A, alcohol **4** has been treated with freshly prepared 2-phenylethanethioic *S*-acid to get diastereomers **15** and **16** in approximately 1:2 ratio. Compound **15** (25 mg) obtained as yellow solid. ^{1}H NMR (400 MHz, CD$_3$OD) δ 7.36 -7.28 (m, 5H, CH$_2$C$_6$**H$_5$**), 4.55-4.53 (m, 1H, CH$_2$C**H**OH), 3.84 (s, 2H, COC**H$_2$**C$_6$H$_5$), 3.25-3.23 (d, *J* = 7.6 Hz, 1H, (OH)CHC**H**CONH), 2.31-2.27 (m, 1H, (OH)CHCH$_2$C**H$_2$**C(SCOPh)), 1.99-1.87 (m, 1H, (OH)CHCH$_2$C**H$_2$**C(SCOPh)), 1.85-1.75 (m, 2H, (OH)CHC**H$_2$**CH$_2$C(SCOPh)); ^{13}C NMR (125 MHz, CD$_3$OD) δ 199.9 (S-**C**=O), 181.0 (SC**C**ONHCOCH), 177.1 (SCCONH**C**OCH), 134.5, 131.0, 129.9, 128.8, 73.9 (CH$_2$**C**HOH), 61.0 ((HO)CH**C**HCONH), 50.3 (PhCH$_2$COS**C**CH), 49.7, 35.2, 33.5; HRMS (ESI) *m/z* calculated for $C_{15}H_{15}NO_4S$ $[M+Na]^+$ 328.0614, found 328.0601. RT_{HPLC} = 6.10 min, purity >95%, 25:75 H$_2$O/ MeOH. Compound **16** (45 mg) obtained as white solid. ^{1}H NMR (500 MHz, CD$_3$OD) δ 7.35-7.30 (m, 5H, CH$_2$C$_6$**H$_5$**), 4.52-4.51 (m, 1H, CH$_2$C**H**OH), 3.86 (s, 2H, COC**H$_2$**C$_6$H$_5$), 3.13 (s, 1H, (OH)CHC**H**CONH), 2.21-2.18 (m, 2H, (OH)CHCH$_2$C**H$_2$**C(SCOPh)), 1.91-1.87 (m, 1H, (OH)CHC**H$_2$**CH$_2$C(SCOPh)), 1.69-1.66 (m, 1H, (OH)CHC**H$_2$**CH$_2$C(SCOPh)); ^{13}C NMR (125 MHz, CD$_3$OD) δ 198.9 (S-**C**=O), 179.6 (SC**C**ONHCOCH), 176.7 (SCCONH**C**OCH), 133.0, 129.5, 128.3, 127.2, 74.8 (CH$_2$**C**HOH), 63.6 ((HO)CH**C**HCONH), 58.4 (PhCH$_2$COS**C**CH), 48.6, 33.0, 31.7; HRMS (ESI) *m/z* calculated for $C_{15}H_{15}NO_4S$ $[M+Na]^+$ 328.0614, found 328.0601. RT_{HPLC} = 5.72 min, purity >93%, 25:75 H$_2$O/ MeOH.

4.1.6. S-((3aR,6aS*)-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) ethanethioate* **(17)**

Following general procedure A, compound **3** has been treated with commercially available thioacetic acid to get **17** (71 mg) as white solid. Melting point 120-122 °C. ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.96 (br s, 1H, N**H**), 3.27-3.24 (d, *J* = 8.8 Hz, 1H, CH$_2$C**H**CONH), 2.34 (s, 3H,

C**H**$_3$CO), 2.32-2.24 (m, 2H, CH_2CH_2C**H**$_2$C(SCOCH$_3$)), 1.83-1.54 (m, 4H, C**H**$_2$C**H**$_2$CH$_2$C(SCOCH$_3$)); ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.3 (S-**C**=O), 178.2 (SC**C**ONHCOCH), 178.2 (SCCONH**C**OCH), 59.5 (CH_3COS**C**CH), 54.7 (CH_2**C**HCONH), 36.2 (**C**H$_3$CO), 29.7, 29.6, 24.3; HRMS (ESI) *m/z* calculated for $C_9H_{11}NO_3S$ $[M+Na]^+$ 236.0348, found 236.0352. RT_{HPLC} 4.90 min, purity >90%, 40:60 H_2O/ MeOH

4.1.7. S-((3aS,6S*,6aR*)-6-Hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 2-chlorobenzothioate* **(18)** *& S-((3aR*,6S*,6aS*)-6-Hydroxy-1,3-dioxohexahydrocyclopenta [c]pyrrol-3a(1H)-yl) 2-chlorobenzothioate* **(19)**

Following general procedure A, alcohol **4** has been treated with freshly prepared 2-chlorobenzothioic *S*-acid to get diastereomers **18** and **19** in approximately 1:3 ratio. Compound **18** (26 mg) obtained as white solid. Melting point 173-174 °C; IRυ_{max} (film) 3743, 2925, 2320, 1707, 1515 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 7.72-7.69 (m, 1H, Aromatic-CClCH=CH-CH=C**H**), 7.55-7.53 (m, 2H, Aromatic-CClC**H**=C**H**-CH=CH), 7.45-7.43 (m, 1H, Aromatic-CClC**H**=C**H**-CH=CH), 4.67-4.63 (m, 1H, CH_2C**H**OH), 3.46 (d, *J* = 7.6 Hz, 1H, (OH)CHC**H**CONH), 2.43-2.39 (m, 1H, (OH)CHCH_2C**H**$_2$C(SCOPh)), 2.08-1.99 (m, 2H, (OH)CHC**H**$_2$C**H**$_2$C(SCOPh)), 1.86-1.83 (m, 1H, (OH)CHC**H**$_2$CH$_2$C(SCOPh)); ^{13}C NMR (100 MHz, CD_3OD) δ 196.0 (S-**C**=O), 183.0 (SC**C**ONHCOCH), 179.3 (SCCONH**C**OCH), 139.7, 136.9, 134.6, 134.3, 133.0, 130.9, 76.3 (CH_2**C**HOH), 64.0 ((HO)CH**C**HCONH), 63.3 (PhCOS**C**CH), 37.7, 35.9; HRMS (ESI) *m/z* calculated for $C_{14}H_{12}NO_4ClS$ $[M+Na]^+$ 348.0068, found 348.0061. RT_{HPLC} 4.46 min, purity 90%, 40:60 H_2O/MeOH. Compound **19** (82 mg) obtained as white solid. Melting point 154-158 °C; IRυ_{max} (film) 3743, 2925, 2320, 1707, 1515 cm^{-1}; ^{1}H NMR (200 MHz, CD_3OD) δ 7.78-7.76 (m, 1H, Aromatic-CClCH=CH-CH=C**H**), 7.58-7.50 (m, 3H, Aromatic-CClC**H**=C**H**-C**H**=CH), 4.63-4.60 (td, *J* = 3.7, 1.2 Hz, 1H, CH_2C**H**OH), 3.34-3.32 (m, 1H, (OH)CHC**H**CONH), 2.39-2.31 (m, 2H, (OH)CHCH_2C**H**$_2$C(SCOPh)), 1.97-1.95 (m, 1H, (OH)CHC**H**$_2$CH$_2$C(SCOPh)), 1.82-1.80 (m, 1H, (OH)CHC**H**$_2$CH$_2$C(SCOPh)); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.6 (S-**C**=O), 178.3 (SC**C**ONHCOCH), 176.1 (SCCONH**C**OCH), 135.0, 133.2, 131.3, 131.1, 129.9, 126.9, 75.4 (CH_2**C**HOH), 63.7 ((HO)CH**C**HCONH), 59.2 (PhCOS**C**CH), 33.2, 32.5; HRMS (ESI) *m/z* calculated for $C_{14}H_{12}NO_4ClS$ $[M+Na]^+$ 348.0068, found 348.0061. RT_{HPLC} = 6.70 min, purity >99%, 40:60 H_2O/ MeOH.

4.1.8. S-((3aS,6S*,6aR*)-6-Hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) benzothioate* **(20) &** *S-((3aR*,6S*,6aS*)-6-Hydroxy-1,3-dioxohexahydrocyclopenta[c] pyrrol-3a(1H)-yl) benzothioate* **(21)**

Following general procedure A, alcohol **4** has been treated with commercially available Thiobenzoic acid to get diastereomers **20** and **21** in approximately 1:2 ratio. Compound **20** (26 mg) as white solid. Melting point 185-186 ºC; IRυ_{max} (film) 3743, 2927, 2320, 1741, 1706, 1531 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 7.91-7.89 (dd, *J* = 8.4, 1.1 Hz, 2H, 2H,2 x Aromatic-*ortho*C**H**), 7.66-7.64 (m, 1H, Aromatic-*para* C**H**), 7.53-7.49 (m, 2H, 2 x 2 x Aromatic-*meta* C**H**), 4.62-4.59 (m, 1H, CH_2C**H**OH), 3.42 (d, *J* = 7.6 Hz, 1H, (OH)CHC**H**CONH), 2.42-2.39 (dd, *J* = 13.2, 7.1 Hz, 1H, (OH)CHCH_2C**H**$_2$C(SCOPh)), 2.08-2.02 (m, 2H, (OH)CHC**H**$_2$C**H**$_2$C(SCOPh)), 1.84-1.82 (m, 1H, (OH)CHC**H**$_2$CH_2C(SCOPh)); ^{13}C NMR (100 MHz, CD_3OD) δ 193.6 (S-**C**=O), 181.1 (SC**C**ONHCOCH), 177.2 (SCCONH**C**OCH), 137.4, 135.6, 130.3, 128.4, 73.9 (CH_2**C**HOH), 61.1 ((HO)CH**C**HCONH), 60.6 (PhCOS**C**CH), 35.3, 33.7; HRMS (ESI) *m/z* calculated for $C_{14}H_{13}NO_4S$ $[M+Na]^+$ 314.0457, found 314.0456. RT_{HPLC} = 6.02 min, purity >90%, 25:75 H_2O/ MeOH. Compound **21** (47 mg) as white solid. Melting point 180-182 ºC; IRυ_{max} (film) 3743, 2927, 2320, 1741, 1706, 1531 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 7.94-7.91 (m, 2H, 2 x Aromatic-*ortho* C**H**), 7.69-7.66 (m, 1H, Aromatic-*para*CH), 7.54-7.51 (m, 2H, 2 x Aromatic-*meta* C**H**), 4.59-4.58 (m, 1H, CH_2C**H**OH), 3.34-3.42 (m, 1H, (OH)CHC**H**CONH), 2.40-2.33 (m, 2H, (OH)CHCH_2C**H**$_2$C(SCOPh)), 1.96-1.95 (m, 1H, (OH)CHC**H**$_2$CH_2C(SCOPh)), 1.78-1.77 (m, 1H, (OH)CHC**H**$_2$CH_2C(SCOPh)); ^{13}C NMR (100 MHz, CD_3OD) δ 191.1 (S-**C**=O), 178.1 (SC**C**ONHCOCH), 175.27 (SCCONH**C**OCH), 134.21, 132.54, 127.23, 125.36, 73.36 (CH_2**C**HOH), 62.28 ((HO)CH**C**HCONH), 56.75 (PhCOS**C**CH), 31.75, 30.32; HRMS (ESI) *m/z* calculated for $C_{14}H_{13}NO_4S$ $[M+Na]^+$ 314.0457, found 314.0456. RT_{HPLC} = 10.8 min, purity >88%, 40:60 H_2O/ MeOH.

4.1.9. S-((3aR,6aS*)-1,3-Dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 4-fluorobenzothioate* **(22)**

Following general procedure A, compound **3** has been treated with freshly prepared 4-fluorobenzothioic *S*-acid to get **22** (40 mg) as white solid. Melting point 186-187 °C; IRυ_{max} (film) 3744, 2922, 1770, 1647 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.40 (br s, 1H, N**H**) 7.96-7.89 (m, 2H, 2 x Aromatic-CC**H**=CH-CF), 7.18-7.08 (m, 2H, 2 x Aromatic-CCH=C**H**-CF), 3.39-3.35 (m, 1H, CH_2C**H**CONH), 2.48-2.28 (m, 2H, CH_2CH_2C**H**$_2$C(SCOPh)), 2.09-1.91 (m, 2H,

C**H**$_2$CH$_2$CH$_2$C(SCOPh)), 1.64-1.59 (m, 2H, CH$_2$C**H**$_2$CH$_2$C(SCOPh)); ^{13}C NMR (100 MHz, $CDCl_3$) δ 190.6 (S-**C**=O), 178.3 (SC**C**ONHCOCH), 178.2 (SCCONH**C**OCH), 167.6, 165.1, 131.9, 130.1, 130.0, 116.1, 116.0, 59.5 (PhCOS**C**CH), 55.0 (CH$_2$**C**HCONH), 36.5, 29.8, 24.5 (CH$_2$**C**H$_2$CH$_2$C(SCOPh)); HRMS (ESI) *m/z* calculated for $C_{14}H_{12}NO_3FS$ $[M+Na]^+$ 316.0414, found 316.0408.

4.1.10. S-((3aR,6aS*)-1,3-Dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 2-chlorobenzothioate* **(23)**

Following general procedure A, compound **3** has been treated with freshly prepared 2-chlorobenzothioic *S*-acid to get **23** (31 mg) as white solid. Melting point 188-190 °C; IRυ$_{max}$ (film) 3229, 1707, 1675, 1547 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.88 (br s, 1H, N**H**), 7.72-7.70 (d, *J* = 7.8 Hz, 1H, Aromatic-CClCH=CH-CH=C**H**), 7.44-7.43 (m, 2H, Aromatic-CClC**H**=C**H**-CH=CH), 7.35-7.33 (m, 1H, Aromatic-CClCH=CH-C**H**=CH), 3.44-3.42 (d, *J* = 8.8 Hz, 1H, CH$_2$C**H**CONH), 2.41-2.38 (m, 1H, CH$_2$CH$_2$C**H**$_2$C(SCOPh)), 2.32-2.30 (m, 1H, CH$_2$CH$_2$C**H**$_2$C(SCOPh)), 1.95-1.93 (m, 1H, C**H**$_2$CH$_2$CH$_2$C(SCOPh)), 1.92-1.90 (m, 2H, C**H**$_2$C**H**$_2$CH$_2$C(SCOPh)), 1.63-1.61 (m, 1H, CH$_2$C**H**$_2$CH$_2$C(SCOPh)); ^{13}C NMR (100 MHz, $CDCl_3$) δ 191.9 (S-**C**=O), 178.1 (SC**C**ONHCOCH), 177.9 (SCCONH**C**OCH), 135.3, 133.1, 131.4, 131.1, 129.7, 126.7, 60.3 (PhCOS**C**CH), 54.8 (CH$_2$**C**HCONH), 36.4, 29.8, 24.5 (CH$_2$**C**H$_2$CH$_2$C(SCOPh)); HRMS (ESI) *m/z* calculated for $C_{14}H_{12}NO_3ClS$ $[M+Na]^+$ 332.0109, found 332.0119. RT_{HPLC} = 7.8 min, purity >92%, 25:75 H_2O/ MeOH.

4.1.11. S-((3aR,6aS*)-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 2-phenylethanethioate* **(24)**

Following general procedure A, compound **3** has been treated with freshly prepared 2-phenylethanethioic *S*-acid to get **24** (80 mg) as sticky liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (br. s., 1H, N**H**), 7.38-7.18 (m, 5H, CH$_2$C$_6$**H**$_5$), 3.82 (s, 4 H, COC**H**$_2$C$_6$H$_5$), 3.21 (d, *J* = 8.8 Hz, 1 H, CH$_2$C**H**CONH), 2.33-2.22 (m, 2H, CH$_2$CH$_2$C**H**$_2$C(SCOPh)), 2.00 - 1.77 (m, 3 H, C**H**$_2$C**H**$_2$CH$_2$C(SCOPh)), 1.91 - 1.70 (m, 1H, CH$_2$C**H**$_2$CH$_2$C(SCOPh)), 1.55 (dd, *J* = 6.1, 12.0 Hz, 1H, CH$_2$C**H**$_2$CH$_2$C(SCOPh)); ^{13}C NMR (100 MHz, $CDCl_3$): δ 198.3 (S-**C**=O), 178.1 (SC**C**ONHCOCH), 178.1 (SCCONH**C**OCH), 132.4, 129.7, 128.9, 127.8, 59.6 (PhCH$_2$COS**C**CH), 54.8 (CH$_2$**C**HCONH), 49.5, 36.3, 29.8, 24.3 CH$_2$**C**H$_2$CH$_2$C(SCOPh); HRMS

(ESI): *m/z* calculated for $C_{15}H_{15}NO_3S$ $[M+Na]^+$ 312.0665, found 312.0657. RT_{HPLC} 3.72 min, purity >90%, 40:60 H_2O/ MeOH.

4.1.12. 6a-((4-Fluorobenzoyl)thio)-1,3-dioxooctahydrocyclopenta[c]pyrrol-4-yl acetate **(25)** Following general procedure A, compound **5** has been treated with freshly prepared 4-fluorobenzothioic *S*-acid to get inseparable mixture of diastereomers of compound **25** (45 mg) as white solid. Melting point 118-119 °C; IRυ_{max} (film) 3743, 3057, 2925, 1707, 1649, 1513 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.58-8.40 (br s, 1H, (S-**C**=O)), 8.00-7.93 (m, 2H, 2 x Aromatic-CC**H**=CH-CF), 7.23-7.14 (m, 2H, 2 x Aromatic-CCH=C**H**-CF), 5.58-5.43 (m, 1H, CH_2C**H**OAc), 3.79-3.55 (m, 1H, (OAc)CHC**H**CONH), 2.51-2.32 (m, 2H, (OAc)CHCH_2C**H**$_2$C(SCOPh)), 2.20-2.11 (m, 4H, (OAc)CHC**H**$_2$CH_2C(SCOPh)), 1.96-1.87 (m, 1H, (OAc)CHC**H**$_2$CH_2C(SCOPh)); ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.0 (S-**C**=O), 177.7, 177.6 (SC**C**ONHCOCH), 174.0, 169.9 (SCCONH**C**OCH), 169.4, 165.4 (CH_3**C**OO), 131.7, 130.2, 130.1, 116.2, 116.0, 73.6 (CH_2**C**HOAc), 60.9 ((AcO)CH**C**HCONH), 58.6 (PhCOS**C**CH), 58.3, 55.3, 33.6, 31.8, 30.4, 29.9, 29.6, 21.0 (**C**H_3CO), 20.8 (**C**H_3CO); HRMS (ESI): *m/z* calculated for $C_{16}H_{14}NO_5SF$ $[M+Na]^+$ 374.0456, found 374.0469. RT_{HPLC} = 6.96 min, purity >90%, 25:75 H_2O/ MeOH.

4.1.13. 6a-((2-Chlorobenzoyl)thio)-1,3-dioxooctahydrocyclopenta[c]pyrrol-4-yl acetate **(26)** Following general procedure A, compound **5** has been treated with freshly prepared 2-chlorobenzothioic *S*-acid to get inseparable mixture of diastereomers of compound **26** (35 mg) obtained as white solid. Melting point 90-91 °C; IRυ_{max} (film) 3744, 1771, 1707, 1547, 1626 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.77 (br s, 1H, N**H**), 7.74-7.73 (d, *J* = 7.6 Hz, 1H, -CClCH=CH-CH=C**H**), 7.47-7.37 (m, 2H, Aromatic-CClC**H**=C**H**-CH=CH), 7.36-7.33 (m, 1H, Aromatic-CClCH=CH-C**H**=CH), 5.55-5.54 (m, 1H, CH_2C**H**(OAc)), 3.57-3.56 (m, 1H, (OAc)CHC**H**CONH), 2.46-2.43 (m, 1H, (OAc)CHCH_2C**H**$_2$C(SCOPh)), 2.26-2.24 (m, 1H, (OAc)CHCH_2C**H**$_2$C(SCOPh)), 2.16-2.13 (dd, *J* = 6.6, 4.7 Hz, 1H, (OAc)CHC**H**$_2$CH_2C(SCOPh)), 2.11 (s, 3H, C**H**$_3$CO), 2.09-1.91 (m, 1H, (OAc)CHC**H**$_2$CH_2C(SCOPh)); ^{13}C NMR (100 MHz, $CDCl_3$): δ 191.9 (S-**C**=O), 177.2 (SC**C**ONHCOCH), 173.6 (SCCONH**C**OCH), 169.9 (CH_3**C**OO), 134.9, 133.3, 131.6, 131.3, 129.9, 126.9, 73.6 (CH_2**C**HOAc), 60.8 ((AcO)CH**C**HCONH), 59.0 (PhCOS**C**CH), 55.2, 33.4, 31.7, 30.4, 30.0, 21.0 (**C**H_3CO), 20.9 (**C**H_3CO); HRMS (ESI): *m/z* calculated for $C_{16}H_{14}NO_5ClS$ $[M+Na]^+$ 390.0173, found 390.0158. RT_{HPLC} = 6.98 min, purity >96%, 25:75 H_2O/ MeOH.

4.1.14. 6a-(Benzoylthio)-1,3-dioxooctahydrocyclopenta[c]pyrrol-4-yl acetate **(27)**

Following general procedure A, compound **5** has been treated with freshly prepared Thiobenzoic acid to get inseparable mixture of diastereomers of compound **27** (25 mg) obtained as white solid. IRυ_{max} (film) 3743, 2922, 1737, 1707, 1675, 1546, 1208 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.05 (br s, 1H, N**H**), 7.92-7.86 (m, 2H, 2 x Aromatic-*ortho* C**H**), 7.64-7.60 (m, 1H, Aromatic-*para* C**H**), 7.49-7.45 (m, 2H, 2 x Aromatic-*meta* C**H**), 5.55-5.44 (m, 1H, CH_2C**H**(OAc)), 3.76-3.54(s, 1H, (OAc)CHC**H**CONH), 2.49-2.47 (m, 1H, (OAc)CHCH_2C**H**$_2$C(SCOPh)), 2.29-2.14 (dt, *J* = 13.4, 6.9 Hz, 1H, (OAc)CHCH_2C**H**$_2$C(SCOPh)), 2.10 (s, 3H, C**H**$_3$CO), 2.11-2.06 (s, 1H, (OAc)CHC**H**$_2$CH_2C(SCOPh)), 1.96-1.93 (m, 2H, (OAc)CHC**H**$_2$CH_2C(SCOPh)); ^{13}C NMR (100 MHz, $CDCl_3$): δ 192.2 (S-**C**=O), 177.7 (SC**C**ONHCOCH), 174.0 (SCCONH**C**OCH), 169.9 (CH_3**C**OO), 135.3, 134.4, 128.9, 127.5, 73.7 (CH_2**C**HOAc), 61.0 ((AcO)CH**C**HCONH), 58.2 (PhCOS**C**CH), 55.4, 33.6, 31.8, 30.4, 30.1, 29.7, 21.1 (**C**H_3COO), 20.8 (**C**H_3COO); HRMS (ESI): *m/z* calculated for $C_{16}H_{15}NO_5S$ $[M+Na]^+$ 356.0553 found 356.0563. RT_{HPLC} = 6.77 min, purity >98%, 25:75 H_2O/ MeOH.

4.1.15. S-((3aR,6S*,6aS*)-6-Hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 4-fluorobenzothioate* **(28)**

Following general procedure A, alcohol **4** has been treated with freshly prepared 4-fluorobenzothioic *S*-acid to get **28** (50 mg) as brown solid. Melting point 184-186 °C; IRυ_{max} (film) 3314, 2934, 1798, 1024 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 8.02-7.98 (m, 2H, Aromatic-CC**H**=CH-CF), 7.29-7.24 (m, 2H, CCH=C**H**-CF), 4.59-4.58 (m, 1H, CH_2C**H**OH), 3.34-3.28 (m, 1H, (OH)CHC**H**CONH), 2.40-2.33 (m, 2H, (OH)CHCH_2C**H**$_2$C(SCOPh)), 1.98-1.95 (m, 1H, (OH)CHC**H**$_2$CH_2C(SCOPh)), 1.84-1.69 (m, 1H, (OH)CHC**H**$_2$CH_2C(SCOPh)); ^{13}C NMR (100 MHz, CD_3OD) δ 191.1 (S-**C**=O), 179.5 (SC**C**ONHCOCH), 176.7 (SCCONH**C**OCH), 167.6, 132.2, 132.2, 129.8, 129.7, 115.8, 115.6, 74.8 (CH_2**C**HOH), 63.7 ((HO)CH**C**HCONH), 58.3 (PhCOS**C**CH), 33.2, 31.8; HRMS (ESI) *m/z* calculated for $C_{14}H_{12}NO_4SF$ $[M+Na]^+$ 332.0363 found 332.0361. RT_{HPLC} = 6.17 min, purity >94%, 40:60 H_2O/ MeOH.

4.1.16. S-((3aR,6S*,6aS*)-6-Hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) thiophene-2-carbothioate* **(29)**

Following general procedure A, alcohol **4** has been treated with freshly prepared thiophene-2-carbothioic *S*-acid to get **29** (70 mg) as white solid. IRυ_{max} (film) 3808, 1741, 1706, 1693, 1515 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 7.91-7.87 (m, 2H, Aromatic-C-S-C**H**=CH-C**H**), 7.23-7.20 (dd, *J* = 4.8, 4.0 Hz, 1H, Aromatic-C-S-CH=C**H**-CH), 4.58 - 4.57 (m, 1H, CH_2C**H**OH), 3.31-3.30(d, 1H, (OH)CHC**H**CONH), 2.36-2.30 (m, 2H, (OH)CHCH_2C**H_2**C(SCOPh)), 1.95-1.94 (m, 1H, OH)CHC**H_2**CH_2C(SCOPh)), 1.76-1.73 (m, 1H, (OH)CHC**H_2**CH_2C(SCOPh)); ^{13}C NMR (100 MHz, CD_3OD) δ 182.8 (S-**C**=O), 177.9 (SC**C**ONHCOCH), 175.1 (SCCONH**C**OCH), 138.5, 132.7, 130.7, 126.7, 73.4 (CH_2**C**HOH), 62.3 ((HO)CH**C**HCONH), 56.9 (C_6H_4COS**C**CH), 31.7, 30.3; HRMS (ESI) *m/z* calculated for $C_{12}H_{11}NO_4S_2$ $[M+Na]^+$ 320.0002, found 320.0008. RT_{HPLC} = 6.96 min, purity >91%, 40:60 H_2O/ MeOH.

4.1.17. 1,3-Dioxo-6a-((2-phenylacetyl)thio)octahydrocyclopenta[c]pyrrol-4-yl acetate **(30)**
Following general procedure A, compound **5** has been treated with freshly prepared 2-phenylethanethioic *S*-acid to get inseparable mixture of diastereomers of compound **30** (45 mg) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.61-8.53 (br s, 1H, N**H**), 7.31-7.17 (m, 5H, CH_2C_6**H_5**), 5.39-5.23 (m, 1H, CH_2C**H**(OAc)), 3.72 (s, 2H, COC**H_2**C_6H_5), 3.51-3.27 (m, 1H, (OAc)CHC**H**CONH), 2.28-2.25 (m, 1H, (OAc)CHCH_2C**H_2**C(SCOPh)), 2.15 (s, 3H, C**H_3**CO), 2.01-1.94 (m, 1H, (OAc)CHCH_2C**H_2**C(SCOPh)), 1.70-1.68 (m, 2H, (OAc)CHC**H_2**CH_2C(SCOPh)); ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.4 , 197.9 (CH_2-**C**OS), 177.6, 177.6 (SC**C**ONHCOCH), 173.7 , 172.6 (SCCONH**C**OCH), 170.5, 169.8 (SC**C**ONHCOCH), 132.2, 132.0, 129.8, 129.7, 128.8, 127.8, 73.6 (CH_2**C**HOAc), 60.7 ((AcO)CH**C**HCONH), 58.7, 58.3 ($PhCH_2COS$**C**CH), 55.1, 49.5, 49.3, 33.4, 31.5, 30.3, 29.8, 29.7, 21.0 (**C**H_3COO), 20.8 (**C**H_3COO); HRMS (ESI) *m/z* calculated for $C_{17}H_{17}NO_5S$ $[M+Na]^+$ 370.0720, found 370.0717. RT_{HPLC} = 6.72 min, purity >98%, 25:75 H_2O/ MeOH.

4.1.18. 6a-(Acetylthio)-1,3-dioxooctahydrocyclopenta[c]pyrrol-4-yl acetate **(31)**
Following general procedure A, compound **5** has been treated with thioacetic acid to get inseparable mixture of diastereomers of compound **31** (60 mg) as white solid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.45 (br s, 1 H, N**H**), 5.53 - 5.35 (m, 1H, CH_2C**H**(OAc)), 3.67-3.43 (m, 1H, (OAc)CHC**H**CONH), 2.39-2.37 (m, 4H, C**H_3**COOCHCH_2C**H_2**C(SCOPh)), 2.35-2.09 (m, 4H, C**H_3**COS, (OAc)CHCH_2C**H_2**C(SCOCH_3)), 1.93-1.82 (m, 2H, OAc)CHC**H_2**CH_2C(SCOCH_3)); ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.6, 196.0 (S-**C**=O), 177.8, 177.7 (SC**C**ONHCOCH), 174.0,

172.8 (SCCONH**C**OCH), 170.5, 169.9 (CH_3**C**OO), 73.6 (CH_2**C**HOAc), 60.7 ((AcO)CH**C**HCONH), 58.7, 58.3 (CH_3COS**C**CH), 55.1, 33.3, 3I.4, 30.3, 29.9, 29.7, 29.6, 29.5, 21.0 (**C**H_3COS), 20.8; HRMS (ESI) *m/z* calculated for $C_{11}H_{13}NO_5S$ $[M+Na]^+$ 294.0407, found 294.0402. RT_{HPLC} = 5.03 min, purity >90%, 20:80 H_2O/ MeOH.

4.1.19. 1,3-Dioxo-6a-((thiophene-2-carbonyl)thio)octahydrocyclopenta[c]pyrrol-4-yl acetate **(32)**

Following general procedure A, compound **5** has been treated with freshly prepared thiophene-2-carbothioic *S*-acid to get inseparable mixture of diastereomers of compound **32** (49 mg) as red color solid. Melting point 140-141 °C; IRυ_{max} (film) 3830, 2922, 2853, 1737, 1707, 1646, 1514, 1210 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.78 (br s, 1H, N**H**), 7.80-7.69 (d, *J* = 3.9 Hz, 1H, Aromatic-S-C**H**=CH-CH), 7.70-7.69 (d, *J* = 4.9 Hz, 1H, Aromatic-S-CH=CH-C**H**), 7.15-7.12 (m, 1H, Aromatic-S-CH=CH-C**H**), 5.53-5.52 (d, *J* = 3.9 Hz, 1H, CH_2C**H**(OAc)), 3.56-3.49 (m, 1H, (OAc)CHC**H**CONH), 2.49-2.43 (m, 1H, (OAc)CHCH_2C**H**$_2$C($SCOC_4H_4S$)), 2.26-2.24 (m, 1H, (OAc)CHCH_2C**H**$_2$C($SCOC_4H_4S$)), 2.11-2.09 (m, 4H, C**H**$_3$COOCHC**H**$_2$$CH_2$C($SCOC_4H_4S$)), 2.07-2.06 (m, 1H, (OAc)CHC**H**$_2$$CH_2$C($SCOC_4H_4S$)); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.8 (S-**C**=O), 177.3 (SC**C**ONHCOCH), 173.6 (SCCONH**C**OCH), 169.8 (CH_3C**O**O), 140.0, 134.4, 134.3, 132.5, 128.3, 77.3 (CH_2**C**H(OAc)), 61.0 ((AcO)CH**C**HCONH), 58.5 (C_6H_4COS**C**CH), 55.6, 33.6, 30.4, 30.1, 29.7, 21.1; HRMS (ESI) *m/z* calculated for $C_{14}H_{13}NO_5S_2$ $[M+H]^+$ 340.0302, found 340.0308. RT_{HPLC} = 5.58 min, purity >95%, 20:80 H_2O/ MeOH.

4.1.20. S-((3aR,6S*,6aS*)-6-Hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 2-hydroxybenzothioate* **(33)**

Following general procedure A, alcohol **4** has been treated with freshly prepared 2-hydroxybenzothioic *S*-acid to get **33** (23 mg) as white solid. Melting point 213-216 °C; IRυ_{max} (film) 2935, 2827, 1823, 1448, 1023 cm^{-1}; ^{1}H NMR (400 MHz, CD_3OD) δ 7.85-7.84 (m, 1H, Aromatic-C-C(OH)CH=C**H**-CH=CH), 7.55-7.51 (m, 1H, Aromatic-C-C(OH)CH=CH-CH=C**H**), 6.98-6.95 (m, 2H, Aromatic-C-C(OH)C**H**=CH-C**H**=CH), 4.59 (m, 1H, CH_2C**H**OH), 3.34-3.32 (d, 1H, (OH)CHC**H**CONH), 2.41-2.33 (m, 2H, (OH)CHCH_2C**H**$_2$C(SCOPh)), 2.00-1.96 (m, 1H, (OH)CHC**H**$_2$$CH_2$C(SCOPh)), 1.78-1.75 (m, 1H, (OH)CHC**H**$_2$$CH_2$C(SCOPh)); ^{13}C NMR (100 MHz, CD_3OD) δ 196.0 (S-**C**=O), 179.6 (SC**C**ONHCOCH), 176.6 (SCCONH**C**OCH), 158.9, 136.1, 128.8, 119.7, 119.3,

117.6, 75.0 (CH_2**C**HOH), 63.7 ((HO)CH**C**HCONH), 58.2 (PhCOS**C**CH), 33.1, 31.7; HRMS (ESI) *m/z* calculated for $C_{14}H_{13}NO_5S$ $[M+Na]^+$ 330.0407, found 330.0405. RT_{HPLC} = 4.96 min, purity >97%, 40:60 H_2O/ MeOH.

4.1.21. S-(1,3-Dioxo-2,3,4,5-tetrahydrocyclopenta[c]pyrrol-3a(1H)-yl)ethanethioate **(34)** *& S,S'-(1,3-Dioxohexahydrocyclopenta[c]pyrrole-3a,6(1H)-diyl)diethanethioate* **(40)**

Following general procedure A, compound **6** has been treated with Thioacetic acid to get compounds **34** and **40.** Compound **34** (40 mg) as white solid. Melting point 170-172 °C; IRυ_{max} (film) 3744, 2924, 2854, 1707, 1515, 1462 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.18 (br s, 1H, N**H**), 6.85 (dd, *J* = 4.2, 2.0 Hz, 1H, CH_2CH_2-C**H**=CCONH), 3.16-3.13 (m, 1H, CH_2C**H**$_2$-CH=C), 2.80-2.75 (m, 1H, CH_2C**H**$_2$-CH=C), 2.48-2.37 (m, 2H, (CH_3COSCC**H**$_2CH_2$-CH=C), 2.32 (s, 3H, C**H**$_3$COS); ^{13}C NMR (100 MHz, $CDCl_3$) δ 194.5 (S-**C**=O), 173.1 (SC**C**ONHCOCH), 163.1 (SCCONH**C**OCH), 140.4, 140.3, 63.8 ($CH_3COSCCH$), 36.8, 35.9, 30.4; HRMS (ESI): *m/z* calculated for $C_9H_9NO_3S$ $[M+Na]^+$ 234.0195, found 234.0194. RT_{HPLC} = 4.84 min purity >95%, 30:70 H_2O/ MeOH. Compound **40** (33 mg) as mixture of diastereomers. ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.8 (br s, 1H, N**H**), 4.37-4.35 (m, 1H, (CH_3COS)C**H**CHCONH), 3.27 (s, 1H, (CH_3COS)CHC**H**CONH)), 2.34 (s, 3H, (C**H**$_3$COS)CHCHCONH)), 2.32 (s, 3H, C**H**$_3$COS), 2.10-1.95 (m, 4H, (CH_3COS)CC**H**$_2$C**H**$_2$CH($SCOCH_3$)); ^{13}C NMR (100 MHz, $CDCl_3$) δ 196.3 (S-**C**=O), 193.3 (S-**C**=O), 177.22 (SC**C**ONHCOCH), 174.7 (SCCONH**C**OCH), 60.2 (CH_3COS**C**HCH), 58.7 (CH_3COS**C**CH), 46.3, 34.1, 31.6, 30.7, 29.6; HRMS (ESI) *m/z* calculated for $C_{11}H_{13}NO_4S_2$ $[M+Na]^+$ 310.0178, found 310.0171. RT_{HPLC} = 6.78 min, 6.42min purity >95%, 40:60 H_2O/ MeOH (diastereomers).

4.1.22. S-(1,3-Dioxo-2,3,4,5-tetrahydrocyclopenta[c]pyrrol-3a(1H)-yl) 4-fluorobenzothioate **(35)** *& S,S'-(1,3-Dioxohexahydrocyclopenta[c]pyrrole-3a,6(1H)-diyl) bis(4-fluorobenzothioate)* **(38)**

Following general procedure A, compound **6** has been treated with freshly prepared 4-fluorobenzothioic *S*-acid to get compounds **35** and **38**. Compound **35** (35 mg) obtained as white solid. Melting point 191-192 °C; IRυ_{max} (film) 3159, 2979, 1714, 1661, 1594, 1200 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.97-7.89 (m, 3H, N**H**, 2 x Aromatic-CC**H**=CH-CF), 7.17-7.09 (m, 2H, 2 x Aromatic-CCH=C**H**-CF), 6.92-6.89 (dd, *J* =4.0, 2.0 Hz, 1H, CH_2CH_2-C**H**=CCONH), 3.23-3.14 (m, 1H, CH_2C**H**$_2$-CH=CCONH), 2.87-2.85 (m, 1H, CH_2C**H**$_2$-CH=CCONH), 2.59-2.56

(m, 2H, (PhCOS)CC**H**$_2$CH$_2$-CH=C); ^{13}C NMR (100 MHz, $CDCl_3$) δ 188.9 (S-**C**=O). 173.1 (SC**C**ONHCOCH), 167.3 (SCCONH**C**OCH), 165.3, 163.3, 140.5, 132.4, 130.2, 130.1, 116.1, 115.9, 63.6 (PhCOS**C**CH), 37.2, 36.0; HRMS (ESI) *m/z* calculated for $C_{14}H_{10}NO_3FS$ $[M+Na]^+$ 314.0258, found 314.0250. Compound **38** (35 mg) obtained as sticky liquid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.35 (br s, 1H, N**H**), 7.98-7.92 (m, 4H, 4 x Aromatic-CC**H**=CH-CF), 7.19-7.09 (m, 4H, 4 x Aromatic-CCH=C**H**-CF), 4.62-4.60 (m, 1H, (PhCOS)C**H**CHCONH)), 3.53-3.52 (t, *J* = 1.4 Hz, 1H, (PhCOS)CHC**H**CONH), 2.50-2.29 (m, 1H, (PhCOS)CCH$_2$C**H**$_2$-CH(SCOPh)), 2.29-2.01 (m, 4H, (PhCOS)CC**H**$_2$C**H**$_2$-CH(SCOPh)); ^{13}C NMR (100 MHz, $CDCl_3$) δ 190.5 (S-**C**=O), 188.4 (S-**C**=O), 177.2 (SC**C**ONHCOCH), 174.8 (SCCONH**C**OCH), 167.8, 167.4, 165.2, 164.9, 132.7, 131.7, 131.6, 130.3, 130.2, 130.0, 129.9, 116.2, 116.1, 115.8, 60.7 (PhCOS**C**HCH), 58.7 (PhCOS**C**CH), 46.7, 34.6, 31.5, 29.7; HRMS (ESI) *m/z* calculated for $C_{21}H_{15}NO_4F_2S_2$ $[M+Na]^+$ 470.0285, found 470.0293. RT_{HPLC} 2.33 min, purity >90%, 40:60 H_2O/ MeOH.

4.1.23. S-(1,3-Dioxo-2,3,4,5-tetrahydrocyclopenta[c]pyrrol-3a(1H)-yl)2-phenylethanethioate **(36)** *and S,S'-(1,3-dioxohexahydrocyclopenta[c]pyrrole-3a,6(1H)-diyl) bis(2-phenylethanethioate)* **(41)**

Following general procedure A, compound **6** has been treated with freshly prepared 2-phenylethanethioic *S*-acid to get compounds **36** (30 mg) as white solid, and **41** as sticky liquid. Compound **36**: Melting point 155-158 °C; IRυ_{max} (film) 3200, 2923, 1764, 1693, 1267 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 7.86 (br s, 1H, N**H**) 7.36-7.24 (m, 5H, CH$_2$C$_6$**H**$_5$), 6.84-6.82 (m, 1H, CH$_2$CH$_2$-C**H**=CCONH), 3.81 (s, 2H, COC**H**$_2$C$_6$H$_5$), 3.14-3.00 (m, 1H, CH$_2$C**H**$_2$-CH=CCONH), 2.82-2.67 (m, 1H, CH$_2$C**H**$_2$-CH=CCONH), 2.47-2.36 (m, 2H, PhCH$_2$COS)CC**H**$_2$CH$_2$-C**H**=C); ^{13}C NMR (125 MHz, $CDCl_3$) δ 196.1 (S-**C**=O), 173.2 (SC**C**ONHCOCH), 163.4 (SCCONH**C**OCH), 140.5, 140.4, 132.3, 129.8, 128.8, 127.8, 63.8 (PhCH$_2$COS**C**CH), 50.2, 36.8, 35.9; HRMS (ESI) *m/z* calculated for $C_{15}H_{13}NO_3S$ $[M+Na]^+$ 310.0508, found 310.0501. RT_{HPLC} 3.70 min, purity >90%, 40:60 H_2O/ MeOH. Compound **41**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.86 (br. s., 1H, N**H**), 7.39-7.23 (m, 10H, 2 x CH$_2$C$_6$**H**$_5$), 4.31 (br. s., 1H), 3.82 (d, *J* = 6.1 Hz, 4H, 2 x SC**H**$_2$COC$_6$H$_5$), 3.21 (s, 1H, (C$_6$H$_5$COCH$_2$S)CHC**H**CONH), 2.30 - 2.28 (m, 1H, C$_6$H$_5$COCH$_2$S)CHC**H**$_2$CH$_2$), 2.09-2.00 (m, 2H, (C$_6$H$_5$COCH$_2$S)CHC**H**$_2$C**H**$_2$C(SCH$_2$COC$_6$H$_5$)), 1.91 - 1.87 (m, 1H, (C$_6$H$_5$COCH$_2$S)CHCH$_2$C**H**$_2$C(SCH$_2$COC$_6$H$_5$)); ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.3 (S-CH$_2$**C**=O), 195.5 (S-CH$_2$**C**=O), 177.2 (SC**C**ONHCOCH), 174.7 (SCCONH**C**OCH), 132.9, 132.2, 129.8, 129.6, 128.9, 128.8, 127.9, 127.7, 77.4, 77.1, 76.8, 60.2

($PhCH_2COS\underline{\mathbf{C}}HCH$), 58.7 ($PhCH_2COS\underline{\mathbf{C}}CH$), 50.5, 49.3, 46.5, 34.2, 31.5; HRMS (ESI): *m/z* calculated for $C_{23}H_{21}NO_4S_2$ $[M+H]^+$ 440.0985, found 440.0981. RT_{HPLC} 22.26 min, purity >95%, 15:85 H_2O/ MeOH.

4.1.24. S-(1,3-Dioxo-1,2,3,4,5,6-hexahydrocyclopenta[c]pyrrol-4-yl) benzothioate **(37)** *& S,S'-(1,3-Dioxohexahydrocyclopenta[c]pyrrole-3a,6(1H)-diyl) dibenzothioate* **(39)**

Following general procedure A, compound **6** has been treated with Thiobenzoic acid to get compounds **37** and **39**. Compound **37** (30 mg) obtained as white solid. Melting point 149-151 °C; IRυ_{max} (film) 3806, 2922, 1707, 1676, 1532 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.94 (dd, *J* = 8.4, 1.3 Hz, 2H, 2 x Aromatic-*ortho* C**H**), 7.64-7.43 (m, 3H, 3 x Aromatic C**H**), 7.23 (br s, 1H, N**H**), 5.09-5.02 (m, 1H, C=CC**H**($SCOC_6H_5$)), 3.28-3.21 (m, 1H, CONH)CC$\underline{\mathbf{H}}_2CH_2CH(SCOC_6H_5)$), 2.88-2.80 (m, 2H, (CONH)CC$\underline{\mathbf{H}}_2C\underline{\mathbf{H}}_2CH(SCOC_6H_5)$), 2.61-2.50 (m, 1H, (CONH)$CCH_2C\underline{\mathbf{H}}_2CH(SCOC_6H_5)$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 190.2 (S-$\underline{\mathbf{C}}$=O), 165.5 (SC$\underline{\mathbf{C}}$ONHCOCH), 164.7 (SCCONH$\underline{\mathbf{C}}$OCH), 156.5, 151.4, 136.2, 133.9, 128.8, 127.4, 41.5 (PhCOS$\underline{\mathbf{C}}$H), 38.3, 25.7; HRMS (ESI) *m/z* calculated for $C_{14}H_{11}NO_3S$ $[M+Na]^+$ 296.0352, found 296.0345. Compound **39** (35 mg) obtained as sticky liquid. ^{1}H NMR (200 MHz, $CDCl_3$) δ 8.45 (br s, 1H, N**H**), 7.98-7.87 (m, 4H, Aromatic-*ortho* C**H**), 7.50-7.47 (m, 2H, 2 x Aromatic-*para* C**H**), 7.46-7.41 (dd, *J* = 7.5, 1.7 Hz, 4H, 2 x Aromatic-*meta* C**H**), 4.63-4.61 (m, 1H, ($C_6H_5COCH_2S$)C$\underline{\mathbf{H}}CH_2CH_2$), 3.53 (s, 1H, ($C_6H_5COCH_2S$)CHC$\underline{\mathbf{H}}$CONH)), 2.51-2.14 (m, 4H, (C_6H_5COS)CHC$\underline{\mathbf{H}}_2C\underline{\mathbf{H}}_2C(SCH_2COC_6H_5)$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 192.1 (S-$\underline{\mathbf{C}}$=O), 190.0 (S-$\underline{\mathbf{C}}$=O), 177.4 (SC$\underline{\mathbf{C}}$ONHCOCH), 174.9 (SCCONH$\underline{\mathbf{C}}$OCH), 136.4, 135.3, 134.4, 133.8, 128.9, 128.7, 127.6, 127.5, 60.7 (PhCOS$\underline{\mathbf{C}}$HCH), 58.6 (PhCOS$\underline{\mathbf{C}}$CH), 46.6, 34.6, 31.6.

4.1.25. S-(1,3-Dioxo-1,2,3,4,5,6-hexahydrocyclopenta[c]pyrrol-4-yl)thiophene-2-carbothioate **(42)**

Following general procedure A, compound **6** has been treated with freshly prepared thiophene-2-carbothioic *S*-acid to get compounds **42** (38 mg) as white solid and traces of dimerised product. Melting point 138-139 °C; IRυ_{max} (film) 2921, 2853, 1709, 1647, 1461 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.79 (d, *J* = 3.9 Hz, 1H, Aromatic-S-C$\underline{\mathbf{H}}$=CH-CH), 7.69-7.65 (m, 1H, Aromatic-S-CH=CH-C$\underline{\mathbf{H}}$), 7.16-7.11 (m, 2H, N$\underline{\mathbf{H}}$, Aromatic-S-CH=C$\underline{\mathbf{H}}$-CH), 5.03-4.96 (m, 1H, C=CC$\underline{\mathbf{H}}$($SCOC_6H_4S$)), 3.30-3.16 (m, 1H, (CONH)CC$\underline{\mathbf{H}}_2CH_2CH(SCOC_6H_4S)$), 2.85-2.79 (m, 2H, (CONH)CC$\underline{\mathbf{H}}_2C\underline{\mathbf{H}}_2CH(SCOC_6H_4S)$), 2.76-2.51 (m, 1H,); ^{13}C NMR (100 MHz, $CDCl_3$):

δ 182.1 (S-**C**=O), 165.5 (SC**C**ONHCOCH), 164.8 (SCCONH**C**OCH), 156.6, 151.1, 141.0, 133.5, 131.8, 128.1, 41.7 ($C_6H_4COS\mathbf{C}H$), 38.3, 25.8; HRMS (ESI): *m/z* calculated for $C_{12}H_9NO_3S_2$ $[M+Na]^+$ 301.9916, found 301.9910. RT_{HPLC} 13.32 min, purity 87%, 10:90 H_2O/ MeOH.

4.1.26. S-((3aR,6S,6aS)-6-Hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 4-fluorobenzothioate **((+)-28b)** *& S-((3aS,6S,6aR)-6-hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 4-fluoro benzothioate* **((-)-28a)**

Following general procedure A, compound (–)-**4** has been treated with freshly prepared 4-fluorobenzothioic *S*-acid to get compounds (+)-**28b** (58 mg) and (–)-**28a (**13 mg**)** as white solids in 5:1 diastereomeric ratio with 51% overall yield, which were carefully separated using silica gel column chromatography. Compound (+)-**28b**: $[\alpha]_D^{30.2}$= +111.4 (*c* = 0.93, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.98 (dd, *J* = 5.2, 8.9 Hz, 2H), 7.24 (t, *J* = 8.5 Hz, 2H), 4.56 (d, J = 3.1 Hz, 1H), 3.27 (s, 1H), 2.49 - 2.23 (m, 2H), 1.94 (br. s., 1H); ^{13}C NMR (101MHz, CD_3OD) δ 191.1, 179.5, 176.7, 167.6, 165.1, 132.2, 132.2, 129.8, 129.7, 115.9, 115.6, 74.8, 63.7, 58.3, 33.2, 31.8; HRMS (ESI): *m/z* calculated for $C_{14}H_{12}NO_4SF$ $[M+Na]^+$ 332.0363 found 332.0367. Compound (–)-**28a:** $[\alpha]_D^{25.5}$= –32.3 (*c* = 0.85, MeOH); ^{1}H NMR (400MHz, CD_3OD) δ 8.03 - 7.97 (m, 2 H), 7.28-7.24 (m, 2 H), 4.63-4.60 (m, 1H), 3.42-3.36 (m, 1H), 2.43-2.40 (m, 1H), 2.22 - 1.94 (m, 2H), 1.87-1.82 (m, 1H); HRMS (ESI): *m/z* calculated for $C_{14}H_{12}NO_4SF$ $[M+Na]^+$ 332.0363 found 332.0365.

4.1.27. S-((3aS,6R,6aR)-6-Hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 4-fluorobenzothioate **((-)-28b)** *& S-((3aR,6R,6aS)-6-hydroxy-1,3-dioxohexahydrocyclopenta[c]pyrrol-3a(1H)-yl) 4-fluorobenzothioate* **(**(+)-**28a)**

Following general procedure A, compound (+)-**4** has been treated with freshly prepared 4-fluorobenzothioic *S*-acid to get compounds (–)-**28b** (63 mg) and (+)-**28a** (14 mg) as white solids in approx 6:1 diastereomeric ratio which were carefully separated using silica gel column chromatography. Compound ((–)-**28b**): $[\alpha]_D^{30.2}$= – 138.4 (*c* = 0.42, MeOH); ^{1}H NMR (400 MHz, CD_3OD) δ 7.97 (dd, *J* = 5.2, 8.9 Hz, 2 H), 7.24 (t, *J* = 8.9 Hz, 2H), 4.62 - 4.49 (m, 1 H), 3.27 (s, 1 H), 2.39 - 2.30 (m, 2 H), 1.97 – 1.92 (m, 1 H); 1.78 - 1.72 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD) δ 192.4, 168.9, 166.4, 133.5, 133.4, 131.1, 131.0, 117.2, 116.9, 76.1, 65.0, 59.7, 34.5, 33.1. Compound ((+)-**28a**): ^{1}H NMR (400MHz, CD_3OD) δ 8.03 - 7.97 (m, 2 H), 7.28 - 7.24 (m,

2 H), 4.63 - 4.60 (m, 1H), 3.42 - 3.36 (m, 1H), 2.43 - 2.40 (m, 1H), 2.22 - 1.94 (m, 2 H), 1.87 - 1.82 (m, 1H).

4.1.28. (3aR,4R,6aS)-6a-((4-Fluorobenzoyl)thio)-1,3-dioxooctahydrocyclopenta[c]pyrrol-4-yl acetate **((-)-25b)**

Following general procedure C, compound (–)-**25b** (45 mg) was obtained as white solid in 97% yield from (–)-**28b**. $[\alpha]_D^{25}$= – 141.7 (*c* = 0.42, $CHCl_3$); ^{1}H NMR (400MHz, $CDCl_3$) δ = 8.81 (br. s., 1H), 7.92 (dd, *J* = 5.5, 7.9 Hz, 2 H), 7.13 (t, *J* = 8.2 Hz, 2H), 5.52 (d, *J* = 3.1 Hz, 1H), 3.51 (s, 1H), 2.49- 2.44 (m, 1H), 2.32 - 2.24 (m, 1 H), 2.19 - 2.10 (m, 1H), 2.06 (s, 3H), 1.92 - 1.86 (m, 2H); ^{13}C NMR (100MHz, $CDCl_3$) δ 190.6, 177.4, 173.8, 169.9, 167.7, 165.2, 131.7, 130.2, 130.1, 116.2, 116.0, 61.0, 58.3, 33.6, 30.4, 21.1. RT_{HPLC} = 5.72 min, purity >99%, 15:85 H_2O/ MeOH.

4.1.29. (3aS,4S,6aR)-6a-((4-Fluorobenzoyl)thio)-1,3-dioxooctahydrocyclopenta[c]pyrrol-4-yl acetate **((+)-25b)**

Following general procedure C, compound (+)-**25b** (46 mg) was obtained as white solid in 98% yield from (+)-**28b**. $[\alpha]_D^{25}$= +124.8 (*c* = 0.42, $CHCl_3$); ^{1}H NMR (400MHz, $CDCl_3$) δ 8.98 - 8.92 (m, 1H), 7.94 (dd, *J* = 5.5, 7.9 Hz, 2H), 7.15 (t, *J* = 8.5 Hz, 2H), 5.54 (d, *J* = 3.1 Hz, 1H), 3.53 (s, 1H), 2.48 (dd, *J* = 7.3, 13.4 Hz, 1H), 2.34 - 2.26 (m, 1H), 2.16 - 2.12 (m, 1H), 2.08 (s, 3H), 1.96 - 1.86 (m, 2H); ^{13}C NMR (100MHz, $CDCl_3$) δ 190.6, 177.5, 173.9, 169.9, 167.7, 165.2, 131.7, 130.2, 130.1, 116.2, 116.0, 77.3, 61.0, 58.3, 33.6, 30.4, 21.1. HRMS (ESI): *m/z* calculated for $C_{16}H_{14}NO_5SF$ $[M+Na]^+$ 374.0469 found 374.0472. RT_{HPLC} = 6.24 min, purity >99%, 15:85 H_2O/ MeOH.

4.1.30. (3aS,4R,6aR)-6a-((4-Fluorobenzoyl)thio)-1,3-dioxooctahydrocyclopenta[c]pyrrol-4-yl acetate **((+)-25a)**

Following general procedure C, compound (+)-**25a** (10 mg) was obtained as white solid in 98% yield from (+)-**28a**. $[\alpha]_D^{25}$= +72.8 (*c* = 0.75, $CHCl_3$); ^{1}H NMR (500 MHz , $CDCl_3$) δ 8.07 (br. s., 1H), 7.93 - 7.91 (m, 2H), 7.16 (t, *J* = 8.2 Hz, 2H), 5.47 - 5.43 (m, 1H), 3.75 (d, *J* = 8.3 Hz, 1H), 2.53 - 2.50 (m, 1H), 2.27 -2.23 (m, 1H), 2.13 (s, 3H), 2.09 - 1.93 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 190.0, 176.8, 171.8, 170.3, 167.5, 165.2, 131.7, 130.2, 130.1, 116.3, 116.1, 73.6, 58.7, 55.5, 31.8, 30.0, 29.7, 20.8. RT_{HPLC} = 5.70 min, purity >98%, 25:75 H_2O/ MeOH.

4.1.31. (3aR,4S,6aS)-6a-((4-Fluorobenzoyl)thio)-1,3-dioxooctahydrocyclopenta[c]pyrrol-4-yl acetate **((–)-25a)**

Following general procedure C, compound (–)-**25a** (13 mg) was obtained as white solid in 90% yield from (–)-**28a**. $[\alpha]_D^{25.3}$ = –79.6 (*c* = 0.42, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.43 (br. s., 1H), 7.93-7.87 (m, 2H), 7.15-7.11 (m, 2H), 5.44-5.39 (m, 1H), 3.73-3.71 (m, 1H), 2.52-2.49 (m, 1H), 2.27-2.23 (m, 1H), 2.13 (s, 3H), 2.09-1.93 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 190.0, 177.1, 172.1, 170.3, 167.5, 165.2, 131.7, 130.2, 130.1, 116.3, 116.1, 73.6, 58.7, 55.5, 31.8, 30.0, 20.8; HRMS (ESI): *m/z* calculated for $C_{16}H_{14}NO_5SF$ $[M+Na]^+$ 374.0469 found 374.0476. RT_{HPLC} 5.40 min, purity >98%, 20:80 H_2O/ MeOH.

4.2. Methodology

4.2.1. Cell culture

Mouse macrophage cell line RAW 264.7was kindly gifted by Prof. ChinmayMukhopadhyay, Special Centre for Molecular Medicine, Jawaharlal Nehru University, India andmaintainedin laboratory at 37°C in RPMI-1640mediasupplemented with 10% heat-inactivated foetal bovine serum (FBS) and penicillin/streptomycin.

4.2.2. Ethics statement

All animal experiments were approved by the Institutional Animal Ethics Committee of the National Brain Research Centre (approval no. NBRC/IAEC/2013/88). The animals were handled in strict accordance with good animal practice as defined by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment and Forestry (CPCSEA), Government of India.

4.2.3. Animal treatment

For *in vivo* experiment, 10 day postnatal BALB/c mouse pups were divided into five groups having five pups irrespective of sex in each group.Among them, three groups were injected intraperitoneally with 10 mg/kg body weight of **25**, **29** and **40** (diluted in 1X PBS)separately for 3 days (once every day). After pre-treatment of drugs, these three groups along with one other group received LPS (5 mg/kg body weight) dissolved in 1X PBS for an additional 24 hours. Control group received the same volume of 1X PBS alone. After 24 hours of LPS treatment, blood was collected from all animals intracardially and serum was prepared.

4.2.4. Cytotoxicity assay

Viability of cultured cells was determinedby-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) as described earlier.[26] RAW 264.7 was seeded in triplicate at a density of 2×10^4 cells per well on a 96-well plate. After 12 h, cells were treated with varying concentrations (0-100 µM) of all the compounds in a serum free condition for another 24 h. MTT solution (0.5 mg/ml) was then added to each well and incubate for 4 h at 37 ºC. At the end of the incubation period, the medium was removed and the resulting purple formazan was solubilizedwith acidic isopropanol (0.1 N HCl in absolute isopropanol), and the absorbance was read at 570 nm using BioradMicroplate reader (Biorad, USA).

4.2.5. Nitric oxide (NO) measurement

Nitrite, a stable oxidized product ofNO, was measured in culture supernatant usingGriess reagent (Sigma Aldrich) according to a previously reported method.[20] After overnight seeding in 96-well plate (2×10^4 cells/well), RAW 264.7 cell was treated with lipopolysachharide (LPS; Sigma) at a concentration of 1µg/ml along with different doses of compounds (as determined from cytotoxicity assay) in serum-free culture for 24 h. Following treatment, media was collected and centrifuged at 2,000 rpm for 5 min to remove cellular debris. 50µL of this media was then reacted with equal volume of Griess reagent for 15 min at room temperature in dark and absorbance was taken at 540 nm using Microplate reader (Biorad, USA). Nitrite concentrations were determined using standard solutions of sodium nitrite prepared in cell culture medium.

4.2.6. Reactive Oxygen Species (ROS) measurement

Intracellular ROS generation in control and treated cells was assessed using the cell permeable, non-polar H_2O_2 sensitive dye 5-(and-6)-chlromethyl-2′, 7′- dichlorodihydrofluorescein diacetate (CM-H2DCFDA) (Sigma Aldrich, USA) as described previously.[27] The extent to which H_2O_2 is generated is defined as the extent of ROS generation. Briefly, RAW 264.7 cells were incubatedwith LPS at concentration of 1µg/ml along with different doses of compound (as determined from cytotoxicity assay) in serum-free culture for 24 h. Upon treatment, the cells were further treated with H2DCFDA (5 µM) for 1 h at 37°C. Cells were washed twice with 1× PBS and fluorescent intensity of the cells was measured using Varioskan flash multimode plate reader.

4.2.7. Immunoblotting

After the treatment with LPS (1 μg/ml) in the presence or absence of compounds (50μM) for 24 h, RAW 264.7 cells were washed with ice-cold PBS and protein is extracted according to an earlier reported method.[28] Protein levels were determined by BCA method. For immunoblot, 20 μgprotein of each samples was separated on (7-10%) polyacrylamide gels, electrophoresed, and transferred onto nitro cellulose membrane. After being blocked with 5% skimmed milk, the membranes were incubated with primary antibodies against COX-2 (1:1000; Millipore, USA), iNOS (1:1000;Millipore,USA), pNF-κβ (1:1000; Cell Signalling Technology, USA) and NF-κβ (1:1000; Santa Cruz Biotechnology, USA). After extensive washes with 0.1% PBS-Tween, blots were incubated with the Anti-Rabbit peroxidase-conjugated secondary antibodies (Vector Laboratories, USA). The blots were processed for development using chemiluminescence reagent (Millipore, USA). The images were captured and analyzed using the Chemigeniusbioimaging system (Syngene, United Kingdom). β-actin antibody (1:10,000; Sigma, USA) was used as loading control.

4.2.8. Cytokine bead array

The cytokine bead array (CBA) kit (BD Biosciences, NJ, USA) was used to quantitatively measure cytokine levels in RAW 264.7cell lysates and mice serum samples. After overnight seeding of cells in 6-well plate, they were treated with LPS along with compounds for 24 h and then cells were harvested for protein isolation. 30 μL of bead mix, containing a population of beads that have been coated with capture antibodies for cytokines, along with equal volume of PE-conjugated detection antibodies were incubated with 30 μg protein samples for 2 h at room temperature in darkandthen the beads were acquired using Cell Quest Pro Software in FACS Calibur and analyzed using BD CBA software (Becton Dickinson, San Diego, CA) as indicated earlier.[29] The mouse serum samples were prepared by centrifuging blood at 2000 rpm for 15 min and CBA was performed as described above.

4.2.9. Statistical analysis

All biological experiments were reproducible andwere performed in triplicate unless otherwise indicated. For analysis of statistical difference, a Student's two-tailed unpaired *t*test was applied. *P* value <0.05 was considered assignificant. The results are expressed as mean ± S.D.

Conflict of interest

No conflict of interest

Notes

[†]These authors contributed equally. The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at
These include copies of ^{1}H and ^{13}C NMR spectra of all new compounds prepared.

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Highlights of paper

1. First synthesis of nitrosporeusines A and B in scalable and green approach.
2. Allylic oxidation, enzymatic resolution and Michael addition are key steps.
3. Synthesized 32 new analogues for initial NO inhibition & selectivity studies.
4. Promising three analogues studied in detail using various inflammatory markers.
5. Isomers of most active compound synthesized & evaluated in *invivo*/*invitro* assays.