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...0.01–...u/ml) challenge. Briefly, 100 1 l of cell culture medium were mixed with 100 1 l of Griess reagent (equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid 0.1% (w/v) naphthylethylendiamine–HCl) and incubated at room temperature for 10 min, and then the absorbance at 550 nm was measured in a Titertek microplate reader (DASIT). Fresh culture medium was used as blank in all the experiments. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve freshly prepared in culture medium. Results are expressed as percentage of inhibition calculated versus cells treated only with LPS.

14. After testing NO release inhibiting activity of compounds 4–8, we decided to test their cytotoxicity (data not shown). Unfortunately, all test derivatives were moderately cytotoxic at concentrations in the range 10^{-4}–10^{-5} M. For this reason, we consider NO release inhibition data at 10^{-6} M more reliable.

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11. Compound 4a: NMR ($CDCl_3$) d 2.72 (s, 3H); 3.54 (s, 3H); 7.46–7.70 (m, 4H); 8.60 (br, 1H).
Compound 4b: NMR ($CDCl_3$) d 1.25–1.28 (t, 3H); 2.66–2.80 (m, 2H); 3.15 (s, 3H); 7.25–7.30 (m, 5H); 8.85 (br, 1H).
Compound 4c: NMR ($CDCl_3$) d 1.47–1.51 (t, 3H); 1.66–1.72 (m, 2H); 2.05–2.18 (m, 2H); 2.83 (s, 3H); 7.43–7.49 (m, 5H); 8.66 (br, 1H).
Compound 4d: NMR ($CDCl_3$) d 1.45–1.53 (m, 6H); 3.61 (s, 3H); 4.43–4.75 (m, 1H); 7.48–7.55 (m, 5H); 8.70 (br, 1H).
Compound 5a: NMR ($CDCl_3$) d 2.00 (s, 3H); 2.40 (s, 3H); 2.52 (d, 3H); 7.10–7.25 (m, 4H); 8.45 (br, 1H).
Compound 5b: NMR ($CDCl_3$) d 1.10–1.15 (t, 3H); 2.01 (s, 3H); 2.41–2.50 (m, 2H); 3.20 (s, 3H); 7.23–7.28 (m, 4H); 8.80 (br, 1H).
Compound 5c: NMR ($CDCl_3$) d 1.49–1.54 (t, 3H); 1.56–1.61 (m, 2H); 1.97 (s, 3H); 2.12–2.22 (m, 2H); 2.76 (s, 3H); 7.45–7.49 (m, 4H); 8.46 (br, 1H).
Compound 5d: NMR ($CDCl_3$) d 1.41–1.49 (m, 6H); 2.00 (s, 3H); 3.76 (s, 3H); 4.49–4.67 (m, 1H); 7.50–7.55 (m, 4H); 8.61 (br, 1H).
Compound 5e: NMR ($CDCl_3$) d 2.02 (s, 3H); 2.43 (s, 3H); 2.62 (d, 3H); 7.15–7.28 (m, 4H); 8.39 (br, 1H).
Compound 5f: NMR ($CDCl_3$) d 1.11–1.15 (t, 3H); 2.12 (s, 3H); 2.41–2.47 (m, 2H); 3.23 (s, 3H); 7.25–7.29 (m, 4H); 8.78 (br, 1H).
Compound 5g: NMR ($CDCl_3$) d 1.48–1.54 (t, 3H); 1.57–1.62 (m, 2H); 1.98 (s, 3H); 2.12–2.22 (m, 2H); 2.78 (s, 3H); 7.45–7.50 (m, 4H); 8.40 (br, 1H).
Compound 5h: NMR ($CDCl_3$) d 1.44–1.51 (m, 6H); 2.14 (s, 3H); 3.77 (s, 3H); 4.47–4.65 (m, 1H); 7.51–7.55 (m, 4H); 8.60 (br, 1H).
Compound 5i: NMR ($CDCl_3$) d 2.39 (s, 3H); 2.63 (s, 3H); 3.64 (s, 3H); 7.46–7.70 (m, 4H); 8.79 (br, 1H).
Compound 5j: NMR ($CDCl_3$) d 1.32–1.37 (t, 3H); 2.86–2.93 (m, 2H); 2.61 (s, 3H); 3.02 (s, 3H); 7.28–7.33 (m, 4H); 8.90 (br, 1H).
Compound 5k: NMR ($CDCl_3$) d 1.50–1.53 (t, 3H); 1.76–1.82 (m, 2H); 2.28–2.32 (m, 2H); 2.60 (s, 3H); 2.80 (s, 3H); 7.40–7.45 (m, 4H); 8.88 (br, 1H).
Compound 5l: NMR ($CDCl_3$) d 1.55–1.57 (m, 6H); 2.61 (s, 3H); 3.54 (s, 3H); 4.43–4.75 (m, 1H); 7.47–7.50 (m, 4H); 8.92 (br, 1H).
Compound 6a: NMR ($CDCl_3$) d 3.17 (s, 3H); 3.52 (s, 3H); 7.56–7.61 (m, 4H); 8.30 (br, 1H).
Compound 6b: NMR ($CDCl_3$) d 2.55 (t, 3H); 3.44 (d, 2H); 3.71 (s, 3H); 7.64–7.71 (m, 4H); 8.42 (br, 1H).
Compound 6c: NMR ($CDCl_3$) d 1.24 (m, 3H); 1.78–1.85 (m, 2H); 2.79 (br, 2H); 3.32 (s, 3H); 7.70–7.80 (m, 4H); 8.00 (br, 1H).
Compound 6d: NMR ($CDCl_3$) d 1.55 (m, 6H); 2.49 (s, 3H); 3.90 (m, 1H); 7.85–7.91 (m, 4H); 8.32 (br, 1H).
Compound 6e: NMR ($CDCl_3$) d 3.10 (s, 3H); 3.32 (s, 3H); 7.49–7.53 (m, 4H); 8.00 (br, 1H).
Compound 6f: NMR ($CDCl_3$) d 2.77 (t, 3H); 3.89 (d, 2H); 3.98 (s, 3H); 7.70–7.81 (m, 4H); 8.00 (br, 1H).
Compound 6g: NMR ($CDCl_3$) d 1.20 (m, 3H); 1.65–1.71 (m, 2H); 2.65 (br, 2H); 3.21 (s, 3H); 7.50–7.80 (m, 4H); 8.04 (br, 1H).
Compound 6h: NMR ($CDCl_3$) d 1.59 (m, 6H); 2.53 (s, 3H); 3.95 (m, 1H); 7.85–7.91 (m, 4H); 8.28 (br, 1H).
Compound 6i: NMR ($CDCl_3$) d 3.19 (s, 3H); 3.48 (s, 3H); 7.59–7.62 (m, 4H); 8.39 (br, 1H).
Compound 6j: NMR ($CDCl_3$) d 2.53 (t, 3H); 3.48 (d, 2H); 3.68 (s, 3H); 7.60–7.65 (m, 4H); 8.40 (br, 1H).
Compound 6k: NMR ($CDCl_3$) d 1.28 (m, 3H); 1.85–1.90 (m, 2H); 2.87 (br, 2H); 3.48 (s, 3H); 7.80–7.85 (m, 4H); 8.39 (br, 1H).
Compound 6l: NMR ($CDCl_3$) d 1.58 (m, 6H); 2.54 (s, 3H); 3.97 (m, 1H); 7.90–7.95 (m, 4H); 8.45 (br, 1H).
Compound 7a: NMR ($CDCl_3$) d 3.19 (s, 3H); 3.51 (s, 3H); 7.57–7.61 (m, 4H); 8.32 (br, 1H).
Compound 7b: NMR ($CDCl_3$) d 2.52 (t, 3H); 3.42 (d, 2H); 3.74 (s, 3H); 7.65–7.71 (m, 4H); 8.40 (br, 1H).
Compound 7c: NMR ($CDCl_3$) d 1.25 (m, 3H); 1.77–1.85 (m, 2H); 2.80 (br, 2H); 3.30 (s, 3H); 7.72–7.79 (m, 4H); 8.01 (br, 1H).
Compound 7d: NMR ($CDCl_3$) d 1.56 (m, 6H); 2.50 (s, 3H); 3.89 (m, 1H); 7.85–7.90 (m, 4H); 8.30 (br, 1H).
Compound 7e: NMR ($CDCl_3$) d 3.12 (s, 3H); 3.33 (s, 3H); 7.51–7.55 (m, 4H); 8.04 (br, 1H).
Compound 7f: NMR ($CDCl_3$) d 2.79 (t, 3H); 3.88 (d, 2H); 3.93 (s, 3H); 7.71–7.79 (m, 4H); 8.01 (br, 1H).
Compound 7g: NMR ($CDCl_3$) d 1.19 (m, 3H); 1.62–1.69 (m, 2H); 2.63 (br, 2H); 3.20 (s, 3H); 7.50–7.76 (m, 4H); 8.00 (br, 1H).
Compound 7h: NMR ($CDCl_3$) d 1.59 (m, 6H); 2.55 (s, 3H); 3.92 (m, 1H); 7.80–7.85 (m, 4H); 8.20 (br, 1H).
Compound 7i: NMR ($CDCl_3$) d 3.12 (s, 3H); 3.35 (s, 3H); 7.49–7.55 (m, 4H); 8.07 (br, 1H).
Compound 7j: NMR ($CDCl_3$) d 2.49 (t, 3H); 3.51 (d, 2H); 3.69 (s, 3H); 7.60–7.67 (m, 4H); 8.43 (br, 1H).
Compound 7k: NMR ($CDCl_3$) d 1.28 (m, 3H); 1.86–1.94 (m, 2H); 2.89 (br, 2H); 3.50 (s, 3H); 7.84–7.89 (m, 4H); 8.49 (br, 1H).
Compound 7l: NMR ($CDCl_3$) d 1.60 (m, 6H); 2.55 (s, 3H); 3.95 (m, 1H); 7.90–7.93 (m, 4H); 8.40 (br, 1H).
Compound 8a: NMR ($CDCl_3$) d 2.61 (s, 3H); 3.60 (s, 3H); 4.55 (s, 2H); 7.40–7.47 (m, 5H); 8.55 (br, 1H).
Compound 8b: NMR ($CDCl_3$) d 1.35–1.39 (t, 3H); 2.71–2.77 (m, 2H); 3.03 (s, 3H); 4.01 (s, 2H); 7.25–7.30 (m, 4H); 8.80 (br, 1H).
Compound 8c: NMR ($CDCl_3$) d 1.50–1.55 (t, 3H); 1.70–1.78 (m, 2H); 2.05–2.12 (m, 2H); 2.79 (s, 3H); 4.60 (s, 2H); 7.50–7.55 (m, 4H); 7.98 (br, 1H, NH).
Compound 8d: NMR ($CDCl_3$) d 1.40–1.47 (m, 6H); 3.61 (s, 3H); 4.15 (s, 2H); 4.45–4.70 (m, 1H); 7.61–7.75 (m, 4H); 8.00 (br, 1H).

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13. The iNOS inhibitory activity of compounds 4–8 was determined (for more details see Marzocco, S.; Piacente, S.; Pizza, C.; Oleszek, W.; Stochmal, A.; Pinto, A.; Sorrentino, R.; Autore, G. Life Sci. 2004, 75, 1491–1501) by measuring nitric oxide release in medium in the presence of each compound. In particular, nitrite accumulation, an indicator of NO release, was detected by Griess reaction (Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. J.; Tannebaum, S. R. Anal. Biochem. 1982, 126, 131–138) in the culture medium of

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ss of iNOS inhibitors (Table 1), and we also report their SAR and pharmacological studies.

Isothioureidic derivatives 4–8[11] were obtained (Scheme 1) starting from properly substituted phenyl isothiocyanates and benzyl isothiocyanates (commercially available) which were condensed with methylamine in acetone to yield the corresponding thioureidic intermediates. The following S-alkylation with the proper alkyl iodide in anhydrous DMF in the presence of potassium carbonate furnished desired compounds 4–8 in good yield.

The model of lipopolysaccharide (LPS)-treated J774.A1 cells is widely used in studies of mechanisms of macrophage iNOS induction, which could be easily tracked down by measurement of accumulation of nitrite in culture medium.[12] Hence, the inhibition of iNOS activity in LPS-activated J774.A1 macrophage cell line of novel isothioureas was evaluated[13] and the results are tabulated below (Table 2).

Most of the compounds exhibited low to moderate capability to inhibit NO release but some of them (4d, 5d, 5h and 5l) were more potent than the reference compound L-NAME (2a). As a general rule, the effect of changing the alkyl substituent on sulfur atom resulted only in slight differences, the activity of S-methyl derivatives being comparable to S-ethyl and S-propyl.

On the contrary, the introduction of the isopropyl group in the same position greatly enhanced the efficacy of the compounds resulting in the most potent derivatives (4d, 5d, 5h and 5l). The introduction of a methyl substituent on the phenyl ring (derivatives 5a–l) enhanced, in most cases, the inhibition of NO release by test compounds in the order ortho > meta > para, at 10^{-6} M (compare, for example, derivatives 4a, 5a, 5e and 5i). However,[14] at 10^{-5} M, the order seems to be para > ortho > meta. A more regular trend emerged from the observation of data for S-isopropyl derivatives, being ortho > meta > para at 10^{-6} M and para > ortho ≈ meta at 10^{-5} M. As a matter of fact, whereas the NO release inhibiting activity of p-methylphenyl substituted S-methyl, S-ethyl and S-propyl compounds (5i, 5j and 5k, respectively) was lower than that of unsubstituted phenyl compounds (4a–c), S-isopropyl derivative (5l) was the most active of tested molecules. This 'para-effect' for S-isopropyl derivatives, suggesting tandem additional binding interactions of compounds with macromolecular target, require supplementary investigations.

On the other hand, the replacement of the aromatic methyl substituent with a fluorine or chlorine atom (compounds 6a–l and 7a–l, respectively) yielded molecules equally or less active than parent compounds. Unexpectedly, the introduction of a methylene linker between the aromatic ring and the isothioureidic moiety increased the amount of nitric oxide formed in LPS-stimulated J774.A1 macrophages after incubation with derivatives 8, even decreasing concentrations down to 0.01 l M.

In conclusion, a series of isothioureidic derivates were synthesised and their inhibitory capability against NO release was evaluated. SAR studies proved the importance of the pharmacophoric isopropyl substituent on the sulfur atom (particularly if coupled with p-methyl substitution on the phenyl ring) S-isopropyl substituted compounds having good activity against the production of nitric oxide in macrophages after stimulation with lipopolysaccharides. Unpredictably, the introduction of a methylene spacer between aromatic and isothioureidic moieties turned the compounds into promoters of NO release. Further investigations on the reasons explaining this phenomena as well as additional assays to explore the activity of test compounds against other (eNOS and nNOS) isoforms are ongoing.

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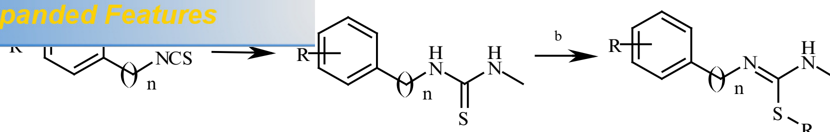

Scheme 1. Reagents and conditions: (a) 4 equiv methylamine, acetone, rt, 8 h; (b) 1.1 equiv R[0]I, K_2CO_3, DMF, rt, 12 h.

More recently, other inhibitors based on guanidines, aminoguanidines, amidines, benzoxazolones, 2-aminopyridines and isothioureas have been reported[9] with various levels of selectivity and potency in vitro.

Our interest in the pharmacological activities of the thiourea moiety[10] prompted us to further investigate this pharmacophore. As a result we herein describe a simple two-step synthesis of 1-methyl-3-phenyl-isothioureas

Table 2. Inhibition of nitrite release by isothioureidic compounds[a]

Compd[b]	Percentage of inhibition at different concentrations[c,d]				
	10^{-3} M	10^{-5} M	10^{-6} M	10^{-7} M[e]	10^{-8} M[e]
4a	65.12 ± 0.23	8.65 ± 3.23	5.10 ± 3.11	—	—
4b	76.76 ± 0.45	2.67 ± 4.23	1.67 ± 0.13	—	—
4c	65.78 ± 4.78	15.70 ± 2.66	4.65 ± 0.34	—	—
4d	81.50 ± 4.88	19.75 ± 0.45	13.21 ± 2.76	—	—
5a	75.10 ± 1.23	17.45 ± 2.31	8.32 ± 0.43	—	—
5b	65.21 ± 3.56	15.33 ± 0.45	7.44 ± 2.31	—	—
5c	81.01 ± 4.22	11.45 ± 1.02	8.12 ± 2.34	—	—
5d	85.21 ± 3.45	18.78 ± 3.89	13.56 ± 2.33	—	—
5e	73.00 ± 1.88	15.34 ± 0.12	7.03 ± 4.21	—	—
5f	81.03 ± 6.32	14.02 ± 2.11	6.79 ± 1.23	—	—
5g	78.34 ± 3.55	12.09 ± 0.22	8.31 ± 5.77	—	—
5h	68.77 ± 2.45	18.55 ± 4.21	19.89 ± 2.55	—	—
5i	67.80 ± 0.12	18.10 ± 1.95	4.67 ± 0.125	—	—
5j	56.82 ± 4.31	0	0	—	—
5k	83.42 ± 2.10	2.08 ± 1.45	1.54 ± 0.21	—	—
5l	73.14 ± 0.22	28.11 ± 4.70	39.80 ± 0.10	—	—
6a	52.45 ± 7.34	6.01 ± 1.84	1.56 ± 3.46	—	—
6b	40.01 ± 0.23	9.76 ± 5.42	3.02 ± 1.23	—	—
6c	65.61 ± 2.22	11.26 ± 5.23	4.15 ± 1.25	—	—
6d	67.71 ± 0.11	9.91 ± 2.33	2.76 ± 1.24	—	—
6e	66.90 ± 4.33	9.34 ± 2.11	5.23 ± 0.54	—	—
6f	59.45 ± 4.78	10.21 ± 5.67	6.23 ± 0.56	—	—
6g	53.91 ± 2.78	11.11 ± 7.43	7.00 ± 0.55	—	—
6h	66.98 ± 6.01	10.04 ± 2.09	4.23 ± 0.65	—	—
6j[b]	60.56 ± 0.45	9.45 ± 0.67	5.98 ± 1.27	—	—
6k	53.45 ± 4.12	10.67 ± 4.03	4.00 ± 5.12	—	—
6l	61.45 ± 1.08	9.03 ± 0.55	4.76 ± 1.33	—	—
7a	21.32 ± 1.95	4.67 ± 2.20	2.90 ± 0.23	—	—
7b	19.55 ± 1.40	10.21 ± 3.22	2.90 ± 4.23	—	—
7c	11.45 ± 4.32	9.55 ± 0.23	1.10 ± 3.21	—	—
7d	16.21 ± 7.34	8.69 ± 1.12	3.21 ± 1.9	—	—
7e	45.38 ± 3.45	12.33 ± 0.45	4.06 ± 1.39	—	—
7f	41.56 ± 1.67	11.56 ± 3.90	3.44 ± 3.20	—	—
7g	58.45 ± 2.22	9.34 ± 0.49	5.01 ± 3.50	—	—
7h	46.90 ± 2.30	8.00 ± 1.04	7.09 ± 2.10	—	—
7i	69.05 ± 4.09	7.55 ± 2.01	2.90 ± 0.12	—	—
7j	54.05 ± 0.98	10.45 ± 3.21	0.50 ± 1.45	—	—
7k	50.04 ± 2.10	5.08 ± 2.08	1.10 ± 0.87	—	—
7l	48.09 ± 3.09	7.09 ± 1.00	8.21 ± 1.04	—	—
8a	—	−54.53 ± 10.88	−9.54 ± 2.18	−5.96 ± 4.77[e]	−2.65 ± 11.46[e]
8b	—	−26.84 ± 12.08	−23.65 ± 6.36	−2.41 ± 7.65[e]	−7.28 ± 8.21[e]
8c[b]	—	−39.58 ± 8.63	−33.71 ± 2.13	−31.36 ± 3.47[e]	−7.96 ± 12.77[e]
L-NAME	69.49	20.37	12.80		

[a] Data are expressed as mean ± s.e.m of at least three experiments, each made in triplicate.
[b] Compounds 6i and 8d precipitated at 10^{-6} M concentration.
[c] Results are expressed as percentage of inhibition calculated versus cells treated only with LPS.
[d] See Ref. 14.
[e] See discussion.

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Synthesis and biological evaluation of 3-benzyl-1-methyl- and 1-methyl-3-phenyl-isothioureas as potential inhibitors of iNOS

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Abstract—Novel benzyl- and phenyl-isothioureidic derivatives have been synthesised and evaluated as inhibitors of nitric oxide synthesis, induced in lipopolysaccharide (LPS)-activated J774.A1 macrophage cell line. The most potent iNOS inhibitor resulting was 1-methyl-3-phenyl-S-methyl isothiourea 5l.
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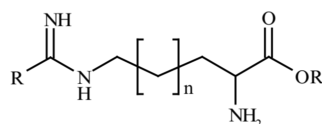
Nitric oxide is a small, highly reactive molecule playing diverse biological roles, depending on which of the three subtypes of the enzyme nitric oxide synthase (NOS) is involved in its biosynthesis from L-arginine and at which location.[1] Two isoforms, endothelial (eNOS) and neuronal (nNOS), are constitutive Ca^{2+}–calmodulin dependent. The third isoform, inducible NOS (iNOS), is formed in response to pathological challenges.

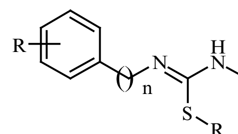
Overexpression of iNOS has been implicated in a number of inflammatory diseases,[2] for example, septic shock, rheumatoid arthritis and platelet aggregation. Selective inhibition of iNOS would therefore be a useful therapy for such diseases, leading to reduction of inflammation, protection of the joint from erosion and possibly alleviation of the associated pain. Indeed, results in animal models of arthritis with non-optimal inhibitors support this hypothesis.[3] However, to date, only one class of compounds has been reported to have entered clinical testing in man.[4]



1.RMe NH;n1;RH
2.RNO $_2$NH;n1;RH(2a RMe)
3. RMe;n2;RH

The best known inhibitors of NOS are amino acids related to the substrate arginine, for example N^x-monomethyl-L-arginine (L-NMMA, 1),[5] N^x-nitro-L-arginine (L-NNA, 2)[6] or its methyl ester L-NAME, 2a, and N-iminoethyl-L-lysine (L-NIL, 3).[7] However, none of them is particularly potent or selective, and cardiovascular effects due to eNOS inhibition[8] limit their usefulness in vivo.

Table 1. New isothioureas iNOS inhibitors



Compd	R	R^0	n
4a–d	H	Me, Et, Pr, i-Pr	0
5a–d	o-CH_3		
5e–h	m-CH_3	Me, Et, Pr, i-Pr	0
5i–l	p-CH_3		
6a–d	o-F		
6e–h	m-F	Me, Et, Pr, i-Pr	0
6i–l	p-F		
7a–d	o-Cl		0
7e–h	m-Cl	Me, Et, Pr, i-Pr	
7i–l	p-Cl		
8a–d	H	Me, Et, Pr, i-Pr	1

Keywords: iNOS; Nitric oxide; Isothioureas.
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