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Investigating the generation of hydrogen sulfide from the phosphonamidodithioate slow-release donor GYY4137

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A combination of NMR spectroscopy, mass spectrometry and chemical synthesis was used to elucidate the two-step hydrolytic decomposition pathway of the slow-release hydrogen sulfide (H_2S) donor GYY4137 and the key decomposition product was also prepared by an independent synthetic route. The (dichloromethane-free) sodium salt of the phosphonamidodithioate GYY4137 was also produced as a pharmaceutically more acceptable salt. In contrast with GYY4137 and its sodium salt, the decomposition product did not generate H_2S or exert cytoprotective or anti-inflammatory effects in oxidatively stressed human Jurkat T-cells and LPS-treated murine RAW264.7 macrophages. The decomposition product represents a useful control compound for determining the biological and pharmacological effects of H_2S generated from GYY4137.

1 Introduction

Following on from the discovery of the biological significance of nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide (H_2S) is the most recent addition to the family of endogenous gasotransmitters.[1-3] Its clinical importance has been highlighted in animal and human studies, where its involvement in diverse processes including blood pressure regulation,[4,5] inflammation,[6] diabetes[7] and neuroprotection[8] has been demonstrated. The amino acids cysteine, homocysteine and cystathionine provide the principal biosynthetic origin of H_2S in mammalian tissues, from pathways catalysed by cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE)[9] and in brain homogenates[10] and the macrovascular endothelium[11] of CBS knock-out mice a third, α-ketoglutarate-and cysteine dependent enzyme, 3-mercaptopyruvate sulfurtransferase (MPST), has also been shown to generate the gasotransmitter. Little is currently known however, as to the possible involvement of the latter pathway in human tissue.

Issues connected with the lack of selectivity for different sulfur-containing species, associated with the common methods for determination of the level of H_2S in blood and its production in tissue, has led to much, as yet unresolved, debate in this area.[2,5] A commonly used spectrometric/HPLC assay based ultimately on the generation of methylene blue from H_2S, hydrosulfide and aqueous sulfide[12] suggests healthy adult plasma serum levels of H_2S to be in the order of 20-60 μM.[5] Plasma concentrations as low as 0.4-0.9 μM have however, been suggested from the results of fluorimetry-based methods.[13] The slow and sustained, enzyme-mediated release of H_2S leads to these low concentrations of the gasotransmitter and it is becoming increasingly clear that for any proposed H_2S donor molecules to give physiologically relevant results, it is very important that they are able to mimic this endogenous process.[2]

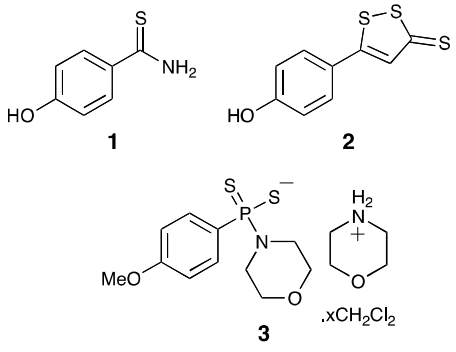


Fig. 1 Slow-release H_2S donors.

In the vast majority of the earlier reported studies, simple sulfide sources such as sodium hydrosulfide (NaSH), sodium sulfide (Na_2S) and saturated aqueous solutions of H_2S were used. Although these facilitate the preparation of solutions of accurately known sulfide concentration, they effectively give an

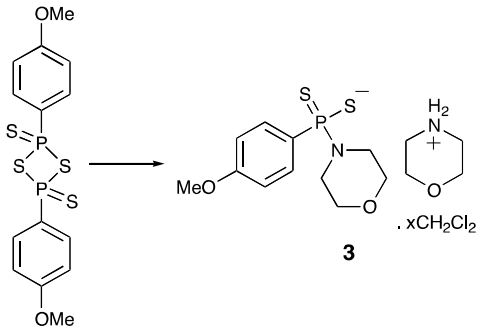
instantaneous delivery of H_2S, which is not representative of the conditions within tissues and living cells.[14,15]

A number of "slow-release" H_2S donors have therefore, been introduced and developed, which give a slow and protracted delivery of sulfide, suitable for *in vivo* experiments.[2,16] Putative H_2S-releasing moities such as 4-hydroxythiobenzamide **1** and 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (ADT-OH) **2** (Fig. 1) have proved successful in this regard, either on their own or in the production of modified versions of existing pharmaceutical products.[17,18] We have also recently reported the preparation and evaluation of a mitochondria-targeted derivative of ADT-OH **2**.[19,20] The phosphonamidodithioate morpholinium salt GYY4137 **3** (Fig. 1) has however, become one of the most widely used slow-release H_2S donor for biological studies.[15] Other structurally related derivatives have also been investigated[21-23] but despite the widespread use of these compounds, surprisingly little has been reported concerning their mode and rate of H_2S release. Also, the nature of the degradation products arising from these compounds after H_2S release have, thus far, only been the subject of speculation and prediction,[16] although their identification is crucial in unravelling the complex physiological and pharmacological effects of these donors.

2 Results and Discussion

2.1 Synthesis of, and hydrolysis pathway for, GYY4137

GYY4137 **3** is precipitated in crystalline form, by the treatment of a dichloromethane solution/suspension of Lawesson's reagent **4** with 4 equiv. of morpholine (Scheme 1).[15,23] The product is obtained as a dichloromethane complex, generally showing a *ca* 2-3 : 1 stoichiometry of GYY4137 **3** to dichloromethane and commercial samples (e.g. SigmaAldrich) also show this composition. An X-ray crystal structure of a dichloromethane-free sample recrystallised from chloroform/petroleum ether confirmed the morpholinium salt structure of **3** (Fig. 2).†



4 3

Scheme 1 Synthesis of GYY4137. *Conditions*: Morpholine (4 equiv.), CH_2Cl_2, 0 °C to RT (45-70%). (x = 0.3 to 0.5)

Although samples of GYY4137 **3** can be stored normally at room temperature in air, the fact that it releases H_2S on contact with water is in no doubt. Samples stored in sealed vials, containing moist air, develop an unmistakeable smell of H_2S after a few days and the headspace gas in the vials causes a rapid blackening of moistened lead(II) acetate-impregnated paper. Aqueous solutions of GYY4137 **3** which have been allowed to decompose, have been used as a control in biological assays[14,28] and Park *et al.* have also observed the decomposition using ^{31}P NMR in acetonitrile containing aqueous HEPES buffer[21] but to the best of our knowledge, neither the actual hydrolysis pathway nor the products formed have been reported previously. Clearly however, these degradation products have the potential to influence the interpretation of any biological results obtained with this H_2S donor and hence, we carried out the experiments described herein, primarily in order to resolve this issue and to produce useful control compounds in a pure state.

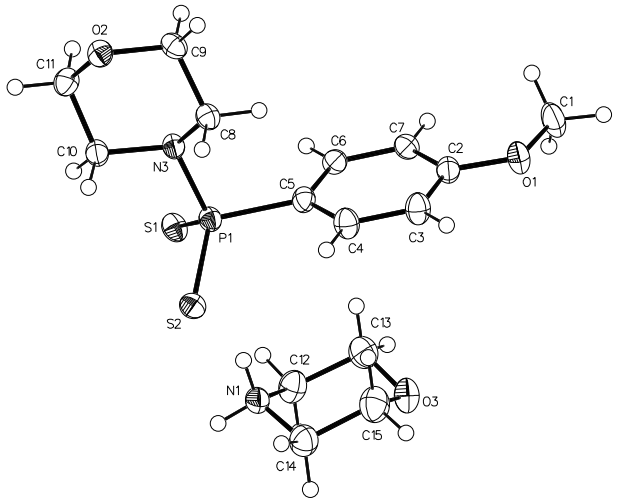
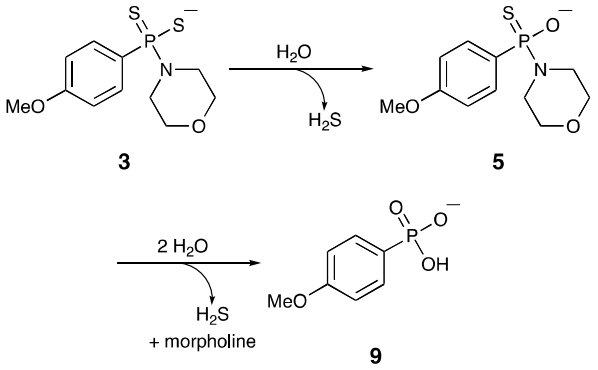


Fig. 2 ORTEP representation of the X-ray crystal structure of GYY4137 **3**. Atomic displacement ellipsoids - 50% probability level.

In line with the results of Park *et al.*,[21] the ^{31}P NMR spectrum of GYY4137 **3** showed a single peak at 90.4 ppm in d_6-acetone and 89.0 ppm in $CDCl_3$ and such values are consistent with the chemical shifts observed for structurally similar arylphosphonamidodithioates.[29] In order to follow the course of the hydrolysis of GYY4137 **3** and ultimately, to determine the phosphorus-containing species produced, a *ca* 1 M solution was prepared in $CDCl_3$ and examined by ^{31}P NMR on a daily basis, relying on adventitious water in the solvent to effect reaction.§ As observed by Park *et al.* when using buffered d_3-acetonitrile as solvent,[21] after 24 h, a clear additional singlet was observed at 64.9 ppm, whose intensity increased steadily with time. The aromatic proton region of the ^1H NMR spectrum also exhibited signals consistent with the formation of a new compound containing a 1,4-disubstituted benzene ring and integration of distinct double-doublets corresponding to GYY4137 **3** and this putative hydrolysis product, suggested a conversion of 10% after 24 h and 14% after 48 h. 50% conversion was achieved in approximately 13 days, with essentially complete loss of the ^{31}P signal corresponding to

GYY4137 **3** after 71 days. (Note: The solution volume in the NMR sample tube was kept constant by regular addition of additional $CDCl_3$, which also ensured the provision of the required small quantities to water to maintain the steady hydrolysis.)

Essentially identical results were obtained using d_6-acetone as solvent, again relying on hydrolysis by adventitious water. A reaction sample from this solvent showing *ca* 50% conversion by ^1H NMR was analysed by mass spectrometry under negative ion electrospray conditions and was found to contain two clear molecular ion peaks. The first at *m/z* = 288.0281 corresponded to the anionic component of GYY4137 **3** with the expected molecular formula of $C_{11}H_{15}NO_2PS_2$ and an identical mass spectrum was obtained for a pure sample of **3**. The second molecular ion was observed at *m/z* = 272.0514, consistent with the replacement of one of the sulfur atoms in the starting material **3** with oxygen, producing a new species with molecular formula $C_{11}H_{15}NO_3PS$. This suggested the formation of compound **5** (Scheme 2) as the initial hydrolysis product of GYY4137 **3** and importantly, its formation would be fully consistent with the formation of H_2S by straightforward sulfur-oxygen exchange. The ^{31}P chemical shift of 64.9 ppm observed for this compound was also consistent with the proposed arylphosphonamidothioate structure **5**.[29] A sample analysed after 71 days of hydrolysis in moist $CDCl_3$ showed only the molecular ion corresponding to this new product **5** under the same mass spectrometry conditions.



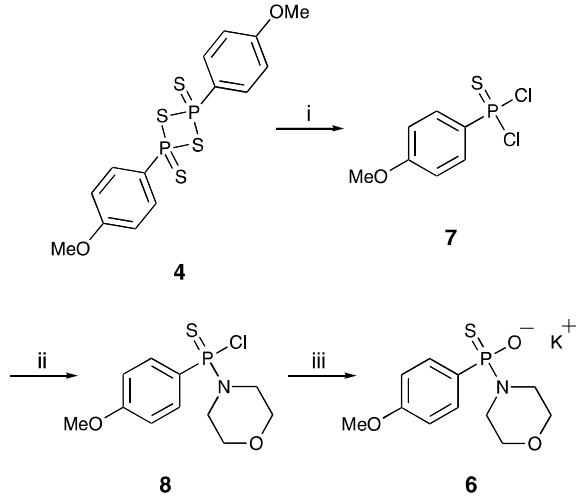
3 **5** **9**

Scheme 2 Two-step hydrolytic degradation of GYY4137 **3**. (Note: Counterion for each structure is morpholinium.)

In order to confirm this structural assignment, we prepared an authentic sample of the potassium salt **6** of the anionic component of **5** by a modification of a previously reported method for the synthesis of this class of compound (Scheme 3).[30] Treatment of Lawesson's reagent **4** with sulfuryl chloride gave phosphonothioic dichloride **7**[31] (72% crude yield), which provided the corresponding morpholinophosphinothioic chloride **8** on reaction with morpholine, in the presence of triethylamine (83% yield).

Reaction of **8** with 2.9 equiv. of potassium trimethylsilanolate in diethyl ether[32] gave a precipitate of pure potassium salt **6** in 94% yield after 7 days. Salt **6** showed a single peak at 64.5 ppm in its ^{31}P NMR spectrum (recorded in D_2O) and a molecular ion of *m/z* = 272.0524, corresponding to the expected molecular formula of the anionic component of $C_{11}H_{15}NO_3PS$.

A solution of **5** in d_6-acetone, produced by hydrolysis of GYY4137 **3**, was carefully layered on top of a similar volume of an approximately equimolar solution of potassium salt **6** in D_2O, with care being taken to avoid initial mixing of the two solutions. A ^{31}P NMR spectrum of this sample of layered solutions revealed two closely-spaced singlets at 62.6 and 63.2 ppm, which coalesced into a single resonance at 65.4 ppm after thorough mixing of the two solutions, confirming the presence of the same phosphorus-containing species in both of the original solutions, as suggested from mass spectrometry.



4 **7** **8** **6**

Scheme 3 Preparation of authentic 4-methoxyphenylphosphonamidothioate **6**. *Reagents and conditions*: (i) SO_2Cl_2 (3.3 equiv.), CCl_4, 0 °C, 1 h then RT, 1 h (72% crude), (ii) morpholine (0.9 equiv.), Et_3N (0.9 equiv.), CH_2Cl_2, RT, 1 h (83% based on morpholine), (iii) $KOSiMe_3$ (2.9 equiv.), Et_2O, RT, 7 days (94%).

These results, in conjunction with ^{13}C NMR spectra, therefore confirmed unambiguously, the structure of the proposed, initial hydrolysis product **5**, resulting from the reaction of GYY4137 **3** with water.

In both of the chloroform and acetone-based hydrolysis solutions described above, a further, minor peak appeared in the ^{31}P NMR spectrum after 7 days at 15.8 ($CDCl_3$) and 13.1 ppm (d_6-acetone). This showed a slight but steady increase in relative intensity in comparison with the ^{31}P signal corresponding to **5** but even after 71 days, this additional product was clearly still a very minor component of the reaction mixture. A negative ion electrospray mass spectrum of the crude product of hydrolysis of GYY4137 **3** after 90 days (in d_6-acetone) revealed, in addition to the peaks corresponding to **5** and a trace of **3**, a third molecular ion at *m/z* = 187.0166, consistent with the molecular formula $C_7H_8O_4P$. This strongly suggests the occurrence of a much slower, extensive hydrolysis of the arylphosphonamidothioate **5** to the phosphonate **9** (with concurrent loss of morpholine and a second equiv. of H_2S) (Scheme 2), the ^{31}P chemical shift observed for this product being in reasonable agreement with that reported for 4-

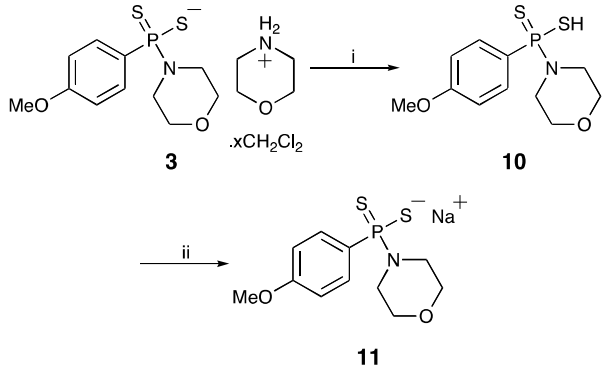
methoxyphenylphosphonic acid. (δ = 15.06 ppm in d_6-DMSO).[33]

The very slow rate of formation of any appreciable quantities of **9** during the hydrolysis process suggests that it is unlikely to make any significant contribution to biological experiments that use GYY4137 **3** as a slow-release H_2S donor. The first hydrolysis product **5** however, clearly has the capability to act as a very slow-release H_2S donor in its own right and hence it and potassium salt **6** represent potentially important and useful control compounds for such studies.

2.2 Preparation of the sodium salt equivalent of GYY4137

The hydrolysis experiments revealed the fact that the phosphorus-bound morpholine is released extremely slowly from GYY4137 **3** and is therefore, unlikely to pose any problems in the use of this compound. The morpholinium counterion however, represents a potential complication to results obtained using this H_2S donor and therefore, in order to eliminate this issue, we also investigated the preparation of a more pharmaceutically acceptable salt. Removal of the dichloromethane from the salt was also a priority and maintaining samples of GYY4137 **3** under high vacuum for long periods of time unfortunately has little, if any, effect on its composition.

Treatment of a cold, aqueous solution of GYY4137 **3** with a 12-fold excess of glacial acetic acid produced a white, powdery precipitate of the phosphinodithioic acid **10** in high (84%) yield (Scheme 4). As expected, **10** proved to be only sparingly soluble in water.



Scheme 4 Synthesis of sodium 4-methoxyphenyl(morpholino)phosphinodithioate **11.** *Reagents and Conditions*: (i) CH_3CO_2H (12 equiv.), H_2O, 0-5 °C (84%). (ii) NaH (1 equiv.), Et_2O, 0 °C to RT (98%).

Addition of sodium hydride to a suspension/solution of **10** in diethyl ether resulted in substantial degradation of the material but portionwise addition of the acid **10** to 1 equiv. of sodium hydride, ensuring that the base was always in excess, gave an excellent (98%) yield of the corresponding (highly water-soluble) sodium salt **11** (Scheme 4). ^{1}H NMR spectra revealed the acid **10** (and therefore, the salt **11**) to be dichloromethane-free.

2.3 H_2S release from GYY4137 and related compounds

H_2S generation from **3**, **6** and **11** was determined using 5,5'-dithiobis-(2-nitrobenzoic acid)[15] (Fig. 3[A]) and resulted in the formation of 2.1 ± 0.6 μM and 2.6 ± 0.3 μM H_2S from **3** and **11** respectively. H_2S was not detected from **6**. Intracellular H_2S was also determined using the sulfide-specific probe 3'-methoxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-6'-yl 2-(pyridin-2-yldisulfanyl)benzoate (WSP-1)[19,34] (Fig. 3[B]) in human Jurkat T-cells. In both assays, **6** failed to generate detectable levels of H_2S when used at the same concentration as **3**, whereas H_2S production was detected from GYY4137 **3** and **11** outside (Fig. 3[A]) and inside (Fig. 3[B]) of cells. These data suggest that **6** may be useful as a control compound for **3** and **11** and for determining whether the biological and pharmacological effects of these compounds are due to H_2S (and/or intermediates that may be formed from them under physiological conditions) from those of the hydrolysis products, such as **6**.

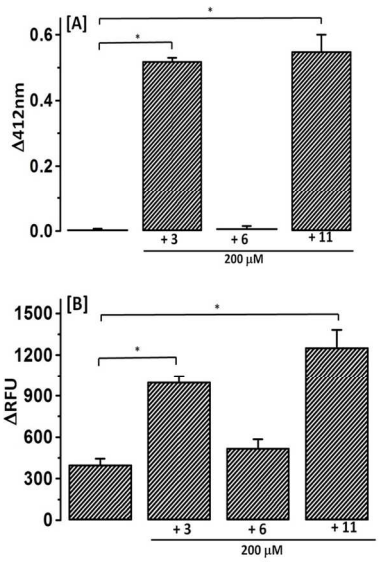


Fig. 3 Generation of H_2S from **3**, **6** and **11. [A]** H_2S generation in the absence of cells using 5,5'-dithiobis-(2-nitrobenzoic acid) and **[B]** intracellular generation of H_2S in human Jurkat cells using WSP-1. Data are mean ± S. D. of six or more determinations. *p < 0.05 *c.f.* oxidant treatment.[‡]

2.4 Effects of compounds 3, 6 and 11 on oxidative stress-induced cytotoxicity

We have shown previously that GYY4137 protected human cells in culture from oxidative stress-induced toxicity**,** induced by 4-hydroxynonenal and SIN-1 but the contribution of the hydrolysis product **6** had not been evaluated.[35] Therefore in order to determine whether **6** would be suitable as a control compound, we exposed human Jurkat T-cells to 4-hydroxynonenal (4-HNE) (20 μM)[34] and SIN-1 (100 μM)**,**[35] in the presence of **3**, **6** and **10** and determined cellular viability after 24 h. Fig. 4 shows that 200 μM **3** and **11** both significantly inhibited oxidative stress-induced cell death. In contrast, no significant inhibition of cell death was observed with **6**, suggesting that the cytoprotective effects of **3** and **11** were due to H_2S. Control experiments showed that **3**, **6**, or **11** alone did

not induce significant cytotoxicity or induce significant cellular proliferation (Trypan blue assay; data not shown).

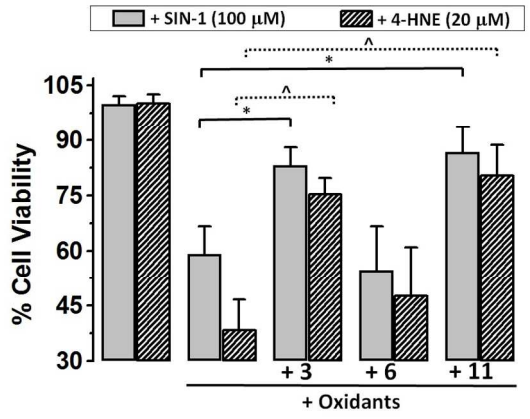


Fig. 4 Inhibition of oxidative-stress induced cytotoxicity by **3**, **6** and **11** in human Jurkat T-cells. Cells were incubated with **3**, **6** or **11** (200 μM) for 1 h and either SIN-1 (100 μM) or 4-HNE (20 μM). Cell viability was assessed by Trypan Blue assay after 24 h. Data are mean ± S. D. of six or more determinations. *p < 0.05 *c.f.* oxidant treatment.[‡]

2.5 Effects of compounds 3, 6 and 11 on inflammation *in vitro*: Nitric oxide (NO) and prostaglandin E_2 (PGE_2) synthesis

Bacterial lipopolysaccharide (LPS) is a well-known inducer of pro-inflammatory enzyme and inducible nitric oxide (NO) synthase and cyclooxygenase-2 in a number of cell types (such as macrophages), leading to the synthesis of NO and PGE_2 respectively. Although GYY4137 has previously been reported to inhibit LPS-induced NO and PGE_2 synthesis, it is not clear whether these effects were due to H_2S (from GYY4137) or from products of GYY4137 hydrolysis such as **6**.[14] To investigate this, we incubated murine RAW264.7 macrophages with LPS (1 μg/ml) in the presence of **3**, **6** and **11** and analysed cell culture media for nitrite by Griess assay (as an index of NO synthesis) and PGE_2 by commercial ELISA. In these studies, significant inhibition of NO (Fig. 5[A]) and PGE_2 (Fig. 5[B]) synthesis were observed with both **3** and **11** but not **6**, suggesting H_2S from **3** and **11** was responsible for the inhibition of NO and PGE_2 synthesis, rather than hydrolysis products such as **6**.

3 Conclusions

In summary, we have elucidated the hydrolysis pathway by which the commonly used slow-release phosphonamidodithioate donor GYY4137 3 produces H_2S. This is two-step process, the first of which involves straightforward sulfur-oxygen exchange with water to give an arylphosphonamidothioate 5 and the second, slower step results in complete hydrolysis to an arylphosphonate **9**. On the normal timescale of biological experiments which employ GYY4137 **3**. as a source of H_2S, the first hydrolysis product 5 is clearly the most significant by-product and its structure was confirmed by an independent synthesis of the arylphosphonamidothioate moiety as its potassium salt **6**. This salt has the potential to be used as a control compound in future evaluation of H_2S donors. The more pharmaceutically acceptable sodium salt equivalent **11** of GYY4137 3 was prepared and characterised and the rates of H_2S generation

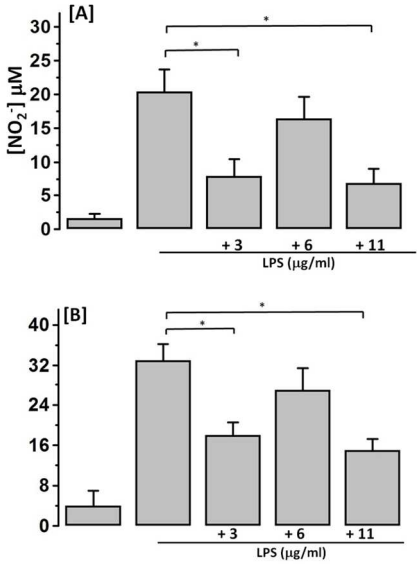


Fig. 5 Inhibition of lipolysaccharide (LPS)-induced nitric oxide and prostaglandin E_2 synthesis by **3**, **6** and **11** in murine RAW264.7 cells. Cells were incubated with **3**, **6** or **11** (200 μM) for 1 h prior to the addition of LPS (1 μg/ml) for 24 h. After this time, cell culture media was collected and assayed for [A] NO_2^- (as an index of nitric oxide generation) and [B] PGE_2 by commercial ELISA. Data are mean ± S. D. of six or more determinations. *p < 0.05 *c.f.* oxidant treatment.[‡]

from **3**, **6** and **11** were monitored using colourimetric and fluorescence assays. Phosphonamidodithioate salts **3** and **11** proved to be equally effective in this regard, whereas generation of the gasotransmitter from hydrolysis product **6** is too low to be significant when compared with the original donors (Fig. 3). Furthermore, **6** failed to prevent oxidant-induced cell death (Fig. 4) and LPS-induced NO and PGE_2 generation (Fig. 5) suggesting **6** may be useful as a control compound in studying the biological effects of **3** and **11** *in vitro* and *in vivo*.

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Notes and references

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† X-Ray crystallographic data of **3** were collected on a Bruker APEXII CCD diffractometer mounted at the window of a Bruker FR591 rotating anode (MoKα, λ = 0.71073 Å) and equipped with an Oxford Cryosystems Cryostream device. Data were processed using the COLLECT package[24] and unit cell parameters were refined against all data. An empirical absorption correction was carried out using SADABS.[25] crystal structure was solved by direct methods and refined on F_o^2 by full-matrix least-squares refinements using SHELX-97 program suite.[26] Graphics were generated using OLEX2.[27] The corresponding CIF has been deposited with the Cambridge has been deposited with the Cambridge Crystallographic Data Centre (Deposition number CCDC 1053548).

Crystallographic data of **3**: a = 24.2051(3) Å, b = 8.9799(2) Å, c = 17.2946(3) Å, α = 90°, β = 101.412(1) °, γ = 90°; V = 3684.82(11) Å^3, Monoclinic, *C*2/*c*, Z = 8, ρ_{calc} = 1.357 Mg/m^3; μ = 0.391 mm^{-1}; *T* = 120(2) K; θ_{max} = 27.47°, 26236 measured reflections, 4230 unique reflections [R_{int} = 0.0416], 3784 with $F^2 > 2\sigma$, *R*(F, $F^2>2\sigma$) = 0.0329; $R_w(F^2$, all data) = 0.0804, GoF = 1.065.

§ The rate of decomposition of aqueous solutions of GYY4137 **3** proved to be too slow to monitor satisfactorily by NMR spectroscopy. Solutions in "wet" organic solvents such as acetone or chloroform however, revealed a substantially faster rate of hydrolysis. We suggest that this may be attributable to an intimate ion pair effect, which renders the phospinodithioate salt in substantially less polar solvents, more reactive towards nucleophilic attack at phosphorus than the solvated anion in pure water.

‡ All graphs are plotted with mean +/- standard deviation. In all cases, the mean values were calculated from data taken from at least six separate experiments. Where significance testing was performed, ANOVA with post-hoc t-test was used; *p < 0.05.

Electronic Supplementary Information (ESI) available: (i) Experimental procedures and characterisation data for the hydrolysis pathway and compounds prepared. (ii) Experimental procedures for cell culture, H_2S generation, cytotoxicity assays and LPS treatment of RAW264.7 cells. See DOI: 10.1039/b000000x/

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