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Phosphorothioate Anti-sense Oligonucleotides: The Kinetics and Mechanism of the Generation of the Sulfurising Agent from Phenylacetyl Disulfide (PADS)

James Scotson[a], Benjamin I. Andrews[b], Andrew P. Laws[a] and Michael I. Page[a]

The synthesis of phosphorothioate oligonucleotides is often accomplished in the pharmaceutical industry by the sulfurisation of the nucleotide-phosphite using phenylacetyl disulfide (PADS) which has an optimal combination of properties. This is best achieved by an initial 'ageing' of PADS for 48 hrs in acetonitrile with 3-picoline to generate polysulfides. The initial base-catalysed degradation of PADS occurs by an $E1_{cB}$-type elimination to generate a ketene and acyldisulfide anion. Proton abstraction to reversibly generate a carbanion is demonstrated by H/D exchange, the rate of which is greatly increased by electron-withdrawing substituents in the aromatic ring of PADS. The ketene can be trapped intramolecularly by an *o*-allyl group. The disulfide anion generated subsequently attacks unreacted PADS on sulfur to give polysulfides, the active sulfurising agent. The rate of degradation of PADS is decreased by less basic substituted pyridines and is only first order in PADS indicating that the rate-limiting step is formation of the disulfide anion from the carbanion.

Introduction

Synthetic oligonucleotides have been used in many different ways for several decades[1], such as therapeutic agents and in diagnostics. An anti-sense oligonucleotide (ASO) is usually a single-stranded deoxy-ribonucleotide (usually 15-35 base pairs in length) which is complementary to a target mRNA. Complex dimer formation between the ASO and the target mRNA through normal Watson-Crick base pairing can lead to inhibition of translation and so prevent synthesis of an unwanted target protein. The formation of the ASO–mRNA hetero-duplex often activates RNase H endonuclease activity which then catalyses the hydrolysis of the mRNA leaving the ASO intact[2]. The first approved anti-sense oligonucleotide was Vitravene (formiversen, for treating the blinding viral condition cytomegalovirus induced retinitis afflicting some of those infected with HIV[3]), which was followed by numerous clinical trials of other oligonucleotides[4] and, more recently, Kynamro (mipomersen) to treat genetically inherited high cholesterol levels[5]. Other mechanisms of action of ASOs include 'exon-skipping' where, instead of binding to mRNA, the ASO binds to longer pre-mRNA chains sterically blocking an unwanted mutation and its surrounding sequence preventing the exon, containing the mutation, being spliced into the mRNA. Antisense-mediated exon skipping is a promising therapeutic for neuromuscular diseases [6], although trials for drisapersen to treat Duchenne Muscular Dystrophy have recently been stopped. ASOs are also useful as valuable genetic diagnostic tools, for example, HyBeacon probes are single-stranded oligonucleotides with one or more internal bases labelled with a fluorescent dye so that when duplex formation occurs with its target sequence there is an increase in fluorescence[7].

However, the use of phosphodiester anti-sense oligonucleotides is limited because they are rapidly hydrolysed by intracellular endonucleases and exonucleases[8]. Consequently, ASOs have been modified to decrease their susceptibility to nuclease cleavage and to increase their uptake into cells, binding efficiency, bio-stability

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and potency[9]. Examples of these modifications are the inclusion of phosphorothioate backbones (**1**)[10] and changes to the 2' position of the ribose sugar (e.g. 2'-O-methoxyethyl) or the sugar-phosphate (e.g. morpholino and locked nucleic acid, (*S*)-constrained-2'-O-ethyl - bicyclic constrained riboses)[11]. These changes do not disrupt H-bonding between the nucleobases.

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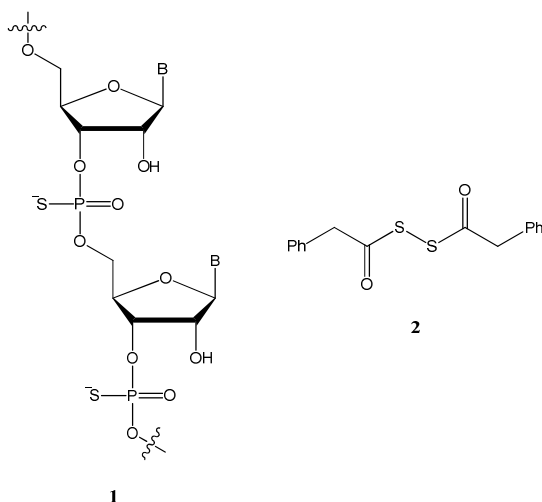
Phosphorothioates (**1**) are the most widely investigated oligonucleotides because of their relative nuclease stability and ease of synthesis and a naturally occurring phosphorothioate has even been found in bacterial DNA[12]. The replacement of one of the phosphate non-bridging oxygens by sulfur introduces chirality at phosphorus and it is only the (*S*)-P phosphorothioate diastereomer that is nuclease resistant[13]. However, phosphorothioate oligonucleotides can reduce the affinity of the ASO for its mRNA target as shown by the melting temperature of the ASO-mRNA hetero-duplex which is decreased by approximately $0.5\,^{\circ}C$ per nucleotide[14]. Conversely, the introduction of the hydrophobic sulfur increases cell uptake compared with the wild-type phosphodiester. Phosphorothioate oligonucleotides remain highly water soluble, still bind well to mRNA and activate RNase H making them the most common modification of ASOs undergoing clinical trials.

The synthesis of phosphorothioate oligonucleotides is often accomplished by the sulfurisation of the nucleotide-phosphite through reaction of the corresponding P(III) analogue, usually attached to a solid support, with an organic sulfurising agent which is present in an organic solvent. As with all the steps involved in the synthesis of oligonucleotide based phosphorthioates, the sulfurisation step of the synthesis should be be rapid, have a near quantitative yield and give a maximal

P=S to P=O ratio. The sulfurisation of phosphorus(III) compounds has been achieved with a number of reagents such as: phenylacetyl disulfide (PADS) (**2**),[15] 3*H*-1,2-benzodithiol-3-one-1,1-dioxide (Beaucage reagent),[16] tetraethylthiuram disulfide (TETD),[17] dibenzoyl tetrasulfide[18], bis-(*O*,*O*-diisopropoxyphosphinothioyl) disulfide (S-Tetra),[19] benzyltriethylammonium tetrathiomolybdate (BTTM)[20], bis-(*p*-toluenesulfonyl) disulfide,[21] 3-ethoxy-1,2,4-dithiazoline-5-one (EDITH) and 1,2,4-dithiazolidine-3,5-dione (DTSNH),[22] bis(ethoxythiocarbonyl)tetrasulfide,[23] 3-methyl-1,2,4-dithiazolin-5-one (MEDITH)[24] and 3-amino-1,2,4-dithiazole-5-thione (ADTT, xanthane hydride).[25]

Although PADS (**2**) has an optimal combination of properties and is often used as the sulfurising reagent in the pharmaceutical industry[26,27], there have been very few mechanistic studies of the sulfurisation reactions of phosphorus (III) analogues using PADS. In particular, PADS must be 'aged' in a basic acetonitrile solution to obtain optimal sulfurisation activity[28] and the reasons for this are not understood. Herein we report the results of our kinetic and mechanistic studies which identify the actual sulfurising agent and suggest an unexpected pathway for its generation. Elsewhere we describe the kinetics and mechanism of the sulfurisation step of substituted triphenyl phosphites and trialkyl phosphites using PADS[29].

Results and Discussion

It is known that a freshly made solution of PADS (**2**) is not an optimal sulfurising agent[26]. Solutions of PADS usually consist of 50% v/v 3-picoline in acetonitrile or other bases such as pyridine or collidine but their efficiency improves greatly upon 'ageing' for about 2 days[26]. PADS degrades completely over this period of time as shown byHPLC, ^{1}H NMR and MS and the colour of the solution changes dramatically from pale yellow through green/blue to brown/black over about 5 days. The pseudo first-order rate constants for the sulfurisation of 0.05 M triphenyl phosphite in acetonitrile by 0.5 M PADS with 1.0 M 3-picoline increase with the length of 'ageing' (**Fig. 1**), such that PADS 'aged' for 48 hrs. is 13-fold more active than a freshly prepared solution of PADS. It is apparent therefore that the degradation product(s) of PADS are more efficient sulfurising agents, but their identity is not known.

The exponential rate of degradation of 3.3 M PADS (**2**) in acetonitrile 25°C was followed by HPLC and the associated first-order rate constants depend on the concentration of 3-picoline (**Fig. 2**). This increase in rate is unlikely to be a consequence of a change
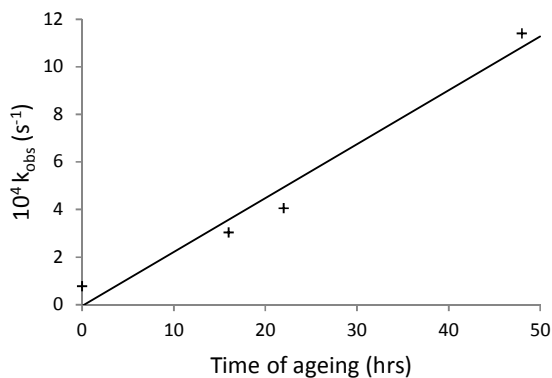

Figure 1 The pseudo first-order rate constants for the sulfurisation of triphenyl phosphite (0.05 M) to $(PhO)_3P=S$ by (0.5 M) PADS (**2**) as a function of the time that PADS has been 'aged' in acetonitrile with (1.0 M) 3-picoline at 25°C

in the dielectric constant of the solution as, for example, PADS is stable in a solution of acetonitrile / dimethylsulfoxide 90/10% v/v over 24 hrs. Furthermore, the rate of degradation of PADS in acetonitrile increases with the basicity of the pyridine base. The slope of the dependence of the observed pseudo first-order rate constants for the decomposition of PADS (**2**) (**Fig. 2**) gives the associated second-order rate constant. These rate constants for the
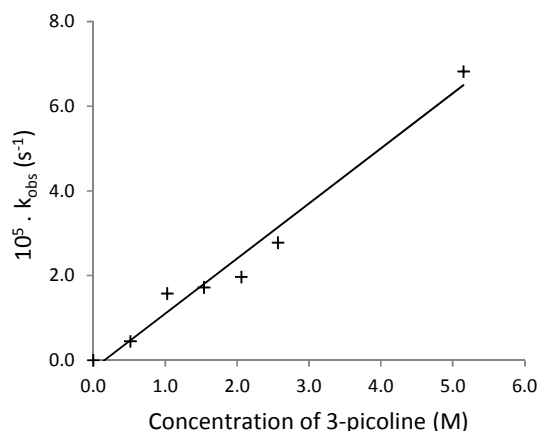

Figure 2 The dependence of the pseudo first-order rate constants for the decomposition of PADS (**2**) in acetonitrile on the concentration of 3-picoline at 25°C.

'ageing' of PADS by substituted pyridines (**Table 1**) generate a Bronsted β-value of 0.37 based on the estimated pK_a in acetonitrile[30]. This is compatible with the pyridine acting as either a base or as a nucleophile with little charge development on the basic nitrogen. The rate of degradation of PADS in acetonitrile is faster with a more basic amine, e.g. with 1.0 M triethylamine it is complete within 5 mins.

Table 1 The second-order rate constants for the decomposition of PADS (**2**) in acetonitrile (ACN) as a function of the basicity of substituted pyridines in ACN at 25°C.

Pyridine substituent	pK_a (ACN) conjugate acid	k_{cat} ($M^{-1}s^{-1}$)
4-MeO	14.73	7.76×10^{-6}
3-Me	13.70	3.34×10^{-6}
H	12.60	1.63×10^{-6}
3-MeO	12.45	3.79×10^{-7}
4-CN	8.50	3.76×10^{-8}
2,6-diMe$_2$	14.72	4.44×10^{-6}

The products of the 'ageing' of PADS are a mixture of diacylpolysulfides and acylpolysulfide anions. HPLC-mass spectroscopy data performed on aged PADS solutions showed the presence of m/z values corresponding to a variety of diacylpolysulfides, in particular the tri-sulfide (**3**) at m/z = 357 (Na^+ adduct) and the penta-sulfide (**4**) at m/z = 437 (K^+ adduct). After several days elemental sulfur crystallised from the solution. The diphenylacetyl polysulfides are presumably formed from nucleophilic attack of the disulfide anion on PADS at sulfur. It is known that disulfide anions are better nucleophiles than the corresponding thiolate[31]. The formation of phenylacetyl disulfide anion (**5**) could result from either nucleophilic attack on PADS or a general-base catalysed elimination reaction of PADS by the picoline base. It is likely that there is an equilibrium set up between the diacylpolysulfides and acylpolysulfide anions and sulfur[32].

3 n = 1   **4** n = 3

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5   **6**
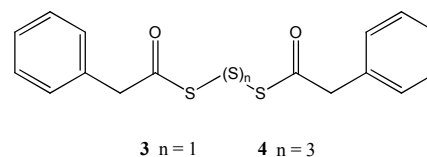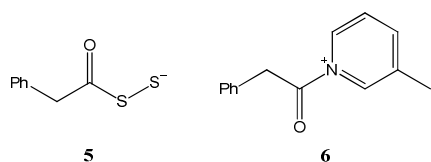
Nucleophilic attack on PADS (**2**) by picoline would generate an acyl-picolinium ion intermediate (**6)** but no direct experimental evidence for this could be found nor could its presence be inferred from trapping experiments with a variety of nucleophiles. However, the general-base catalysed formation of the disulfide anion by an $E1_{cB}$ - type mechanism (**Scheme 1**) is indicated by both D-exchange and trapping of the ketene intermediate.
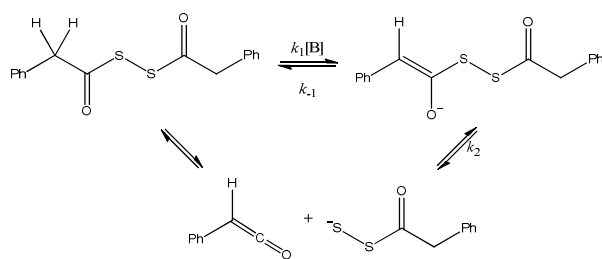
Scheme 1

The rate of H/D exchange was measured by ^{1}H NMR using a solution of 0.17M PADS (**2**) in d_3-acetonitrile containing various concentrations of 3-picoline and 0.6M D_2O at 25°C. The CH_2 singlet at δ 4.15 ppm disappeared with a first-order decay generating a triplet for CHD at δ 4.13 ppm which reached a maximum intensity followed by its disappearance to give CD_2. The pseudo first-order rate constants for D-exchange showed a first-order dependence on the concentration of 3-picoline (**Fig. 3**) which gives a second-order rate constant k_{pic} = 4.72 x 10^{-3} M^{-1}s^{-1}.

The mechanism of D-exchange presumably occurs through the formation of the intermediate carbanion. The relatively low dielectric constant of the solvent may mean that the ionization of this carbon acid may give rise to ion-pairs in equilibrium with the dissociated species (**Scheme 2**), where the product K_iK_d corresponds to the normal ionization constant K_a. However, it is unlikely that exchange can occur from the ion-pair and so if isotopic exchange occurs with the dissociated carbanion, then the rate of protonation of the carbanion k_{-2} can be assumed to be diffusion-controlled[33], which, based on a viscosity of 0.35cP for acetonitrile can be

assumed to be ~ 10^{10} M^{-1}s^{-1}. The pK_a value for PADS in acetonitrile/water (90/10% v/v) can then be calculated to be 26.0
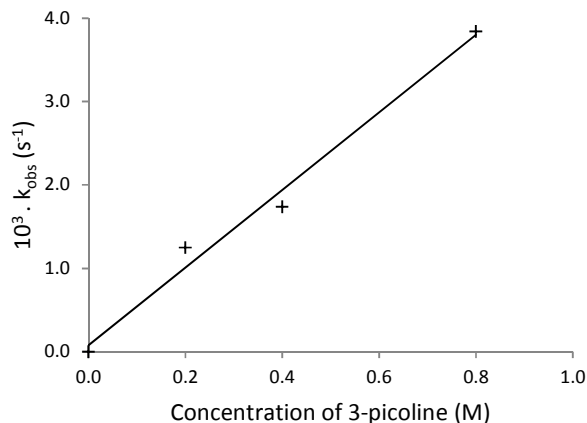
Figure 3 The dependence of the pseudo observed first-order rate constants for the H/D - exchange of PADS on the concentration of 3-picoline in acetonitrile at 25°C.

by using this estimated diffusion-controlled rate constant, the measured rate of D-exchange and the pK_a of 13.7 for 3-picoline in acetonitrile[34]. There are no pK_a data for thioesters in acetonitrile but that for ethyl thioacetate in water is 21[35] and, based on substituent and solvent effects[30], that for ethyl thiophenylacetate $PhCH_2COSEt$ in acetonitrile is ~ 31, so the estimated value of 26.0 for PADS is reasonable given the additional electron-withdrawing $SSCOCH_2Ph$ residue.
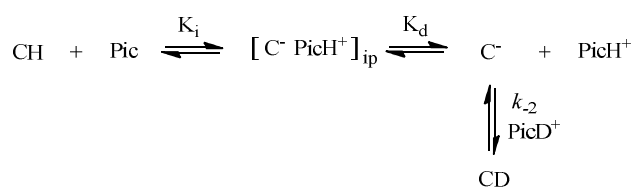
Scheme 2

The rate of degradation of PADS (**2**) is *ca*. 500-fold slower than the rate of D-exchange under the same conditions, indicating that carbanion formation is relevant to the formation of the degradation products of PADS. Indeed, removing the dissociable protons in PADS by α,α dimethylation prevents the degradation of the tetramethyl derivative **7** which remains unchanged after 1 week in acetonitrile with 5 equivalents of picoline at 25°C. Similarly, bis-

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benzoyl disulfide is stable in acetonitrile with 5 equivalents of picoline at 25°C for 1 week, confirming the elimination mechanism for the degradation of PADS (**2**).
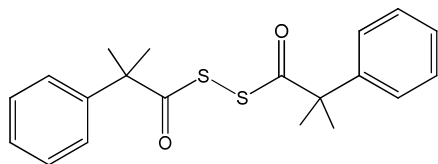
7

The proposed mechanism for the degradation of PADS (**Scheme 1**) involves the generation of the ketene intermediate. The reaction of *o*-allyl PADS (**8**) in 50% v/v acetonitrile/3-picoline yields the bicyclic ketone (**9**) identified by MS and ^1H NMR, consistent with trapping the intermediate ketene by a [2+2] cycloaddition. With PADS itself, the ketene forms the 3-hydroxycyclobutenone dimer, (**10**) and the pyrononone trimer (**11**) as observed with the base-catalysed reaction of phenylacetyl chloride[36].
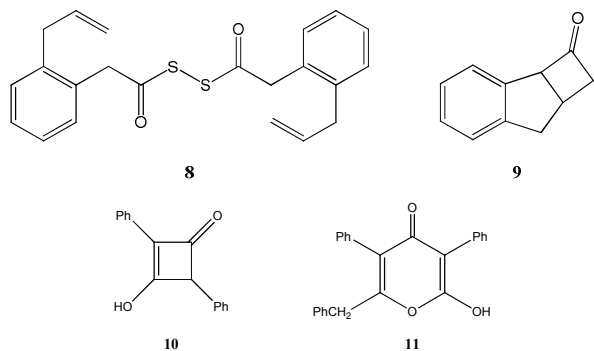
8   **9**

10   **11**

The relative effect of substituents in the aromatic ring of PADS (**2**) on the rate constants for H/D-exchange and the degradation process are shown in **Table 2.**

Table 2 The dependence of the second-order rate constants for H/D-exchange and for the degradation of substituted PADS (**2**) in acetonitrile at 25°C

PADS substituent	σ	k_{ex} ($M^{-1}s^{-1}$)
4-MeO	-0.27	1.38×10^{-3}
H	0	5.16×10^{-3}
4-Cl	0.23	2.20×10^{-2}
4-CN	0.66	1.09×10^{-1}

All of the above observations suggest that a polar mechanism is taking place, which is also supported by the rate of degradation of PADS (**2**) being unaffected by light or dark or the addition of the radical scavenger 2,6-di-*tert*-butyl-4-methylphenol (BHT).

In summary, the degradation of PADS (**2**) in acetonitrile catalysed by pyridine bases involves reversible carbanion formation, which expels acyl disulfide anion to generate a ketene intermediate. The disulfide anion then attacks unreacted PADS at sulfur to give polysulfides (*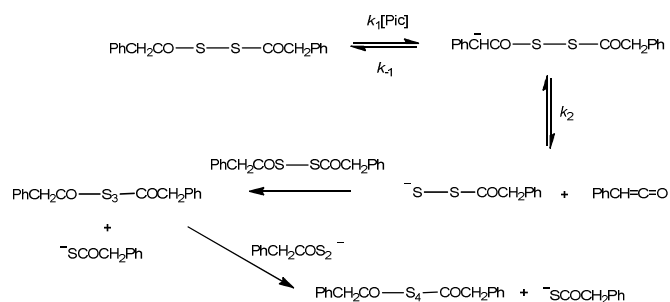*Scheme 3**). Other possibilities are feasible, including polysulfide anion attack on PADS (**2**) and diacylated polysulfides. It is interesting that the rate of degradation is only first order in PADS (**2**) indicating that the rate-limiting step is k_2 formation of the disulfide anion from the carbanion, rather than its subsequent reaction with a second mole of PADS.

Scheme 3

Conclusion

The activation of PADS by its 'ageing' for 48 hrs in acetonitrile with 3-picoline involves the generation of polysulfides which are the actual nucleotide-phosphite sulfurising agents. The initial base-catalysed degradation of PADS occurs by an $E1_{cB}$-type elimination to generate a ketene and acyldisulfide anion. Carbanion formation from PADS is reversible, demonstrated by H/D exchange, the mechanism of which occurs by base catalysis and is greatly increased by electron-withdrawing substituents in the aromatic ring of PADS. The ketene can be trapped intramolecularly by an *o*-allyl group. The disulfide anion generated subsequently attacks unreacted PADS on sulfur to give polysulfides, the active sulfurising agent. The rate of degradation of PADS is decreased by less basic substituted pyridines and is only

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first order in PADS indicating that the rate-limiting step is formation of the disulfide anion from the carbanion.

Experimental

NMR experiments were performed using a 400MHz Bruker Avance DP X400 NMR spectrometer. HPLC traces were acquired using a Shimadzu SIL 50AH instrument with a Luna 5µ C18 4.67x250mm column. The gradient used for kinetic measurements was 30-95% acetonitrile in water over 10 minutes followed by isocratic elution for a further 10 minutes.

H/D exchange of PADS To a solution of phenylacetyl disulfide in deuterated acetonitrile (0.2M, 400 µl) and D$_2$O (50 µl) was added 3-picoline (15.5 µl, 16.2 mg, 0.17mmol). The ^{1}H NMR of this mixture was run every 2 minutes until exchange was complete.

General degradation kinetics of PADS A solution of PADS (1000ppm, 3.3mM) in HPLC-grade acetonitrile containing the appropriate concentration of 3-picoline was divided between 2 HPLC vials and chromatograms were taken every 30 minutes for 48 hours.

General sulfurisation kinetics with 'aged' PADS A solution of PADS (1M) 50/50 v/v acetonitrile/3-picoline was left to age for 48 hours. At T=*n* hrs a 1ml sample of this was removed and quenched with dilute hydrochloric acid (10ml, 2M) to remove the picoline as the protonated pyridinium ion. This was then washed with DCM (2 x 10ml) the combined organic layers dried and the solvent removed under vacuum. The resulting oil was then made up to 1ml using deuterated acetonitrile. To an NMR tube containing triphenyl phosphite in deuterated acetonitrile (0.1M, 200 µL) and 3-picoline in deuterated acetonitrile (2M, 200 µL) was added 'aged' PADS solution (200 µL, 1M) and ^{31}P NMR spectrum recorded every 90 seconds.

Trapping the ketene intermediate A mixture of 2,2'-allyl-2,2'-phenylacetyl disulfide (330mg, 0.852mmol) and 3-picoline (165.6 µl, 1.7mmol) in acetonitrile (10ml) was refluxed for 2 hr. The reaction was followed by HPLC and was complete after 2 hrs. The mass spec and NMR analysis showed evidence of the product 7,7a-dihydro-1H-cyclobuta[a]inden-2(2aH)-one as described below.

Synthesis

Methyl 2'-iodo-2-phenylacetate: 2'-iodo-phenylacetic acid (5.01 g, 19.1 mmol) and H$_2$SO$_4$ (98%, 1.25 ml) were dissolved in methanol (7.5 ml). The reaction mixture was stirred for 2.5 hr at 65°C, then diluted with 250 ml dichloromethane (DCM), extracted with water (2 x 100 ml) and brine (50 ml). The organic solution was dried over MgSO$_4$, and gravity filtered. DCM was removed under vacuum to give a pale yellow oil (4.88 g, 92.6% yield). HPLC retention time 5.397 min; ^{1}H NMR (CDCl$_3$, 400MHz) δ 3.69 (s, 3H, CH$_3$), 3.79 (s, 2H, CH$_2$), 6.92-6.96 (m, 1H, ArH), 7.22-7.32 (m, 2H, ArH), 7.83 (d, 1H, J 7.91Hz, ArH) ppm; ^{13}C NMR (CDCl$_3$, 100MHz) δ 46.12 (CH$_3$), 52.22 (CH$_2$), 101.07 (CI), 128.48 (CH), 128.94 (CH), 130.68 (CH), 137.72 (Cq), 139.54 (CH), 170.95 (CO) ppm; IR (film) 1732.81 cm^{-1}.

Methyl 2'-allyl-2-phenylacetate[37]: methyl 2'-iodo 2-phenylacetate (3.0 g, 10.9 mmol), CsF (6.6 g, 43.6 mmol), and Pd(PPh$_3$)$_4$ (0.63 g, 0.55 mmol) were dissolved in THF (250 ml), stirred for 30 min at room temperature. A solution of allylboronic acid pinacol ester (3.3 ml, 21.8 mmol) in THF (50 mL) was added and then heated under reflux for 24 hr. The reaction mixture was diluted with petroleum ether (bpt. 60–80°C, 300mL), extracted with water (2 x 200ml) and the combined aqueous layers washed with petroleum ether (bpt 60-80°C, 100ml). The combined organic layers were washed with water (100 mL), brine (100 mL), dried over MgSO$_4$. The solvent was removed under vacuum to give an orange oil product (2.5 g) which was purified using a Biotage SP4 chromatography system fitted with a Biotage Snap 10 column eluting with a gradient of 0-25% ethyl acetate in hexane over 15 column volumes to give the product ester as a yellow oil (1.6 g, 77.2% yield). HPLC retention time 5.630 min; ^{1}H NMR (CDCl$_3$, 400MHz) δ 3.39 (dt, 2H, J 6.29Hz, CH$_2$), 3.61 (s, 2H, CH$_2$), 3.65 (s, 3H, CH$_3$), 4.94-5.06 (m, 2H, CH$_2$), 5.87-5.97 (m, 1H, CH), 7.14-7.23 (m, 4H, ArH) ppm; ^{13}C NMR (CDCl$_3$, 100MHz) δ 37.35 (CH$_2$), 38.45 (CH$_2$), 51.94 (CH$_3$), 115.96 (CH$_2$), 126.65 (CH), 127.55 (CH), 129.91 (CH), 130.65 (CH), 132.66 (Cq), 136.57 (CH$_2$), 138.35 (Cq), 171.95 (CO) ppm; IR (film) 1734.52 cm^{-1}.

2'-Allyl 2-phenylacetic acid: to methyl-2'-allyl 2-phenylacetate (1.7 g, 8.9 mmol) in THF (20ml) was added an aqueous lithium hydroxide solution (0.4M, 20ml) and the solution stirred at room temperature for 3 hr after which the mixture was diluted with water (40ml) and extracted with DCM (2 x 20ml). The pH of the aqueous layer was reduced to pH 1 with HCl, extracted with DCM (2 x 20ml), dried over MgSO$_4$ and the solvent removed under vacuum giving the brown oil product (1.7 g, 9.7 mmol, 82.9% yield). HPLC retention time 4.634 min; ^{1}H NMR (CDCl$_3$, 400MHz) δ 3.38 (dt, 2H, J 6.37 Hz, CH$_2$), 3.65 (s, 2H, CH$_2$), 4.94-5.06 (m, 2H, CH$_2$), 5.86-5.96 (m, 1H, CH), 7.14-7.23 (m, 4H, ArH), 10.76 (broad, s, 1H, OH) ppm; ^{13}C NMR (CDCl$_3$, 100MHz) δ 37.5 (CH$_2$), 38.45 (CH$_2$), 116.22 (CH$_2$), 126.79 (CH), 127.96 (CH), 130.08 (CH), 130.88 (CH), 132.05 (Cq), 136.52 (CH), 138.55 (Cq), 178.41 (CO) ppm; IR (film) 1702.49cm^{-1}.

2,2'-Diallyl 2,2-phenylacetyl disulfide (8)[38]: sodium hydrosulfide (798 mg, 14.25 mmol) was dissolved in ethanol (5 ml) and chilled over ice. The flask was fitted with an exhaust line allowing any gasses generated to be scrubbed by sodium hypochlorite in a well-ventilated fume hood. In a separate flask, to 2'-allyl 2-phenylacetic acid (1.0 g, 5.7 mmol) dissolved in DCM (10 ml) was added Ghosez reagent (1.04 g, 1.03 ml, 8.5 mmol) then stirred at room temperature for 30 min. and the solvent removed under vacuum to yield an orange oil. This oil was added to the ethanolic sodium hydrosulfide solution which instantly turned yellow and the precipitate of sodium chloride removed under vacuum filtration and washed with ice cold ethanol (1.5ml). To the solution, stirred over ice, iodine was slowly added until the colour of the suspension changed from white to pale brown (approximately 1 g, 7.8 mmol). The resulting mixture was diluted with DCM (10 ml), washed twice with saturated sodium thiosulfate solution (2 x 15 ml) and the organic layer concentrated under vacuum to yield the brown oil product which was purified using a Biotage SP4 chromatography system fitted with a Biotage Snap 10 column eluting with 25% ethyl acetate in hexane (*Rf*= 0.41) to give the product as a clear oil (384 mg, 1 mmol, 14.1% yield). HPLC retention time 7.6 min; ^{1}H NMR

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(CDCl$_3$, 400MHz) δ 3.41 (d, 4H, J= 6.3 Hz, CH$_2$), 4.00 (s, 4H, CH$_2$), 4.96-5.60 (m, 4H, CH$_2$), 5.88-5.98 (m, 2H, CH), 7.17-7.30 (m, 8H, ArH) ppm; ^{13}C NMR (CDCl$_3$, 100MHz) δ 37.51 (CH$_2$), 46.68 (CH$_2$), 116.63 (CH$_2$), 126.95 (CH), 128.58 (CH), 130.22 (CH), 130.88 (Cq), 131.53 (CH), 136.12 (CH), 139.07 (Cq), 191.81 (CO) ppm.

7,7a-dihydro-1H-cyclobuta[a]inden-2(2aH)-one (9): A mixture of 2′-allyl 2-phenylacetylchloride (100 mg, 0.514 mmol) and 1-chloro-*N,N,2*-trimethyl-1-propenylamine (74.7 µl, 0.56 mmol) in DCM (10 ml) was stirred for 30 min at room temperature, triethylamine (78 µl, 0.56 mmol) in DCM (4 ml) was added and refluxed for 2 hr. The reaction mixture was cooled and extracted with 2M hydrochloric acid (20 ml) to remove the 3-picoline. The crude bright orange oil product mixture was purified using a Gilson prep. HPLC system equipped with a phenomenex luna C18, 5µ 250 x 20 mm column. The mobile phase consisted of a gradient of 50-100% acetonitrile (0.05% TFA) in water (0.05% TFA) over 15minutes then isocratic elution at 50% acetonitrile (0.05% TFA) in water (0.05% TFA) for a further 3 minutes. The product eluted at 7.5 min. All fractions were combined and the solvent removed under vacuum to give the product as a white, crystalline solid (24 mg, 41.8% yield). HPLC retention time 4.775 min; ^{1}H NMR (d$_3$ACN, 400MHz) δ 2 2.75-2.87 (m, 1H, CH$_2$), 3.05 (d, 1H, J 16.73, CH$_2$), 3.11-3.18 (m, 1H, CH), 3.30-3.42 (m, 1H, CH$_2$), 3.38-3.45 (m, 1H, CH$_2$), 4.71 (broad, s, 1H), 7.23-7.36 (m, 4H, ArH); ^{13}C NMR (ACN-d^3, 100MHz) δ 26.18 (CH), 38.82 (CH$_2$), 52.32 (CH$_2$), 71.81 (CH), 124.45 (CH), 125.50 (CH), 126.82 (CH), 127.52 (CH), 137.6 (Cq), 143.38 (Cq), 206.13 (CO), IR (film) 1771.39 cm^{-1}.

2,2,2′,2′-Tetramethyl 2,2′phenylacetyl disulfide (7): Sodium hydrosulfide (4.2 g, 75 mmol) was dissolved in ethanol (30 ml) and chilled over ice. The flask was fitted with an exhaust line allowing any gasses generated to be scrubbed by sodium hypochlorite in a well-ventilated fume hood. In a separate flask, to 2-phenylisobutyric acid (5 g, 30 mmol) dissolved in DCM (20 ml) was added Ghosez reagent (6.16 g, 5.6 ml, 45 mmol) then stirred at room temperature for 30 min. and the solvent removed under vacuum to yield an orange oil. This oil was added to the ethanolic sodium hydrosulfide solution which instantly turned yellow and the precipitate of sodium chloride removed under vacuum filtration and washed with ice cold ethanol (1.5ml). To the solution, stirred over ice, iodine (2 g, 15.6 mmol) was slowly added. The resulting precipitate was collected by vacuum filtration and washed with ice-cold ethanol (5 ml) and water (10ml) to give a waxy yellow solid (2.2 g, 5.9 mmol, 39.3 % yield). HPLC retention time 7.6 min; ^{1}H NMR (CDCl$_3$, 400MHz) δ 1.68 (s, 12H), 7.34 (m, 10H) ppm; ^{13}C NMR (CDCl$_3$, 100MHz) δ 26.82, 54.21, 127.27, 127.86, 128.61, 142.14, 199.39 ppm. HRMS (m/z): [M+NH$_4$]$^+$ for C$_{20}$H$_{22}$O$_2$S$_2$, calculated 390.0782, measured 390.0788; IR: 1707.3 cm^{-1} (film).

2,2′-(4-chlorophenyl)acetyl disulfide: as above using sodium hydrosulfide (1.6 g, 29.25 mmol) in ethanol; (15 ml) 4-chlorophenylacetic acid (2 g, 11.7 mmol) in DCM (20 ml); Ghosez reagent (1.72 g, 1.57 ml, 12.9 mmol); iodine (0.5 g, 3.9 mmol) to give product (1.5 g, 4.1 mmol, 70.1 % yield). HPLC retention time 7.3 min; ^{1}H NMR (CDCl$_3$, 400MHz) δ 3.94 (s, 4H, CH$_2$), 7.17-7.39 7.52 (d,d, 8H, J$_1$ 8.28 Hz, J$_2$ 92.54 Hz, ArH) ppm; ^{13}C NMR (CDCl$_3$, 100MHz) δ 48.37 (CH$_2$), 129.12 (CH), 130.48 (Cq), 131.06 (CH), 134.12 (Cq), 190.83 (CO) ppm. HRMS (m/z): [M+NH$_4$]$^+$ for C$_{16}$H$_{12}$Cl$_2$O$_2$S$_2$, calculated 387.9994, measured 388.0000; IR: 1720.2cm^{-1} (film).

2,2′-(4-cyanophenyl)acetyl disulfide: as above using sodium hydrosulfide (2.4 g, 43 mmol) in ethanol (20 ml); 4-cyanophenylacetic acid (2.753 g, 17.1 mmol) in DCM (20 ml); Ghosez reagent (3.432 g, 3.120 ml, 25.7 mmol); iodine (0.5 g, 3.9 mmol) to give the product as a orange/yellow solid (1.6 g, 4.6 mmol, 53.8 % yield). HPLC retention time 6.0 min; ^{1}H NMR (CDCl$_3$, 400MHz) δ 4.08 (s, 4H), 7.53 (d,d, 8H, J$_1$ 8.06 Hz, J$_2$ 92.53 Hz, ArH) ppm; ^{13}C NMR (CDCl$_3$, 100MHz) δ 48.82 (CH$_2$), 112.06 (CN), 118.41 (Cq), 130.47 (CH), 132.65 (CH), 137.28 (Cq), 189.76 (CO) ppm. HRMS (m/z): [M+NH$_4$]$^+$ for C$_{18}$H$_{12}$N$_2$O$_2$S$_2$, calculated 352.0340, measured 352.0352; IR: 1715.6 cm^{-1} (film).

2,2′-(4-methoxyphenyl)acetyl disulfide: as above using sodium hydrosulfide (4.26 g, 75.3 mmol) in ethanol (20 ml); 4-methoxyphenylacetic acid (5 g, 30.1 mmol) in DCM (20 ml); Ghosez reagent (8.03g, 7.96 ml, 60.2 mmol); iodine (1 g, 7.8 mmol) to give product (1.19 g, 3.28 mmol, 10.9 % yield). ^{1}H NMR (CDCl$_3$, 400MHz) δ 3.79 (s, 6H, CH$_3$), 3.92 (s, 4H, CH$_2$), 7.04 (d,d, 8H, J$_1$ 8.75 Hz, J$_2$ 114.31, ArH) ppm; ^{13}C NMR (CDCl$_3$, 100MHz) δ 48.6 (CH$_3$), 55.2 (CH$_2$), 114.7 (CH), 124.5 (Cq), 131.1 (CH), 159.5 (Cq), 192.5 (CO) ppm; HRMS (m/z): [M+NH$_4$]$^+$ for C$_{18}$H$_{18}$O$_4$S$_2$, calculated 362.0647, measured 362.0634.

Bis-benzoyl disulfide[39]: To *s*odium sulfide (1.84g, 23.6mmol) in water (12ml) and heated to 50°C was added tetra-N-butylammonium bromide (380mg, 1.18mmol) then stirred for 30 minutes. In a separate flask, (fitted with an exhaust line allowing any gasses generated to be scrubbed by sodium hypochlorite in a well-ventilated fume hood) benzoyl chloride (2ml, 1.65g, 11.8mmol) in toluene (12ml). This was chilled in an ice bath. After 30 minutes the sodium sulfide solution was cooled to room temperature and slowly added to the rapidly stirring, chilled benzoyl chloride solution under a nitrogen atmosphere creating a biphasic reaction mixture. This was stirred for 24 hours at room temperature. The organic and aqueous layers were separated, the aqueous layer washed with 2 x 5ml portions of toluene and the organic layers combined. The toluene was then removed under vacuum giving the product as white crystals (738mg, 45.7% yield) from which a single crystal X-ray diffraction pattern was resolved. The aqueous layer contains residual Na$_2$S which will generate H$_2$S if exposed to acid and was disposed of carefully. ^{1}H NMR (CDCl$_3$, 400MHz) δ 7.43 (t, 4H, ArH), 7.57 (t, 2H, ArH), 7.89 (d, 4H, ArH)ppm; ^{13}C NMR (CDCl$_3$, 100MHz) δ 127.91 (CH), 128.79 (CH), 134.02 (CH), 136.61 (Cq), 190.37 (CO) ppm.

Acknowledgements

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