Studies of nitrile oxide cycloadditions, and the phenolic oxidative coupling of vanillin aldoxime by *Geobacillus* sp. DDS012 from Italian rye grass silage†‡

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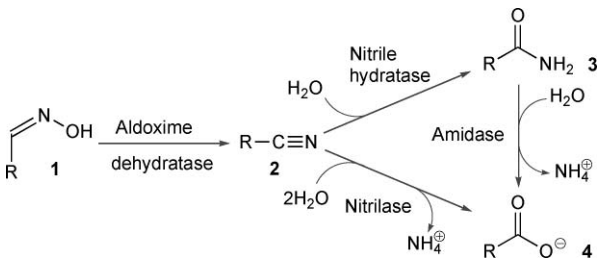
During studies directed towards the discovery of nitrile hydrolysing enzymes from thermophiles, vanillin aldoxime was incubated with the thermophilic organism, *Geobacillus* sp. DDS012 isolated from Italian rye grass (*Lolium multiflorum*) silage. The predominant product was a dihydro-dimer, which could only be characterised by LC-MS. This was initially imagined to be the product of cycloaddition of vanillin aldoxime with the corresponding nitrile oxide, but preparation of the supposed adduct and model studies excluded this possibility. The rate constant for the second order dimerisation of 4-*O*-acetyl vanillin nitrile oxide was measured (1.21×10^{-4} M^{-1} s^{-1}, 0.413 M, 25 °C) and the ^{13}C-NMR signal for the nitrile oxide carbon was observed (δ_C 34.4, br. t $^1J^{13}C,^{14}N$ *circa* 50 Hz). Treatment of vanillin aldoxime with potassium persulfate and iron sulfate gave material with the same LC-MS properties as the natural product, which is therefore identified as 5,5′-dehydro-di-(vanillin aldoxime) **1d** formed by phenolic oxidative coupling.

Introduction

There are two classes of enzymes, which hydrolyse nitriles **2**.¶ Nitrilases (EC 3.5.5.1) hydrolyse nitriles directly to carboxylic acids **4** without forming free amide intermediates, whereas nitrile hydratases (EC 4.2.1.84) give the amide **3**. Almost invariably, the amide **3** so formed is hydrolysed to the carboxylic acid **4** by a co-expressed amidase (EC 3.5.1.4).[1] In microbiological studies prospecting for nitrile hydrolysis activity, nitrile hydratases are found far more commonly than nitrilases. This appears to be a consequence of limited genetic capability, because in a survey of 150 sequenced bacterial genomes, only ten were found to harbour nitrilase genes.[2]



The hydrolysis of nitriles to amides and carboxylic acids requires vigorous conditions with strong acids or bases and separation of the product from spent reagent is expensive. Consequently there has been much interest in developing enzymatic methods for nitrile hydrolysis, which proceed under mild conditions.[3] The most notable success is the *Nitto* process for the hydrolysis of acrylonitrile to acrylamide by *R. rhodochrous* J1 whole cells, which contain a nitrile hydratase that can constitute up to 50% of the soluble protein in the cell. The productivity is greater than 7 Kg of acrylamide per g of dry cells and production is currently *circa* 30 000 tons per annum.[4]

Screening of more than six hundred biotope specific environmental DNA libraries yielded 137 characterised nitrilases. The activity of a few has been optimised for synthetic applications by site-directed saturation mutagenesis, but it remains to be demonstrated how many will be useful synthetically.[5] Screening of environmental samples for nitrile hydrolysis activity with representative nitriles is a powerful selection strategy, which avoids the necessity to develop expensive high speed screening techniques. Unfortunately, nitriles are almost invariably toxic to whole cells reducing the chances of inducing nitrile hydrolysis activity by feeding experiments. We have employed the strategy of feeding aldoximes **1**, which undergo *in situ* dehydration to nitriles, catalysed by aldoxime dehydratase.[6] Our intention is to reduce the nitrile concentration below the toxic threshold[7] and moreover aldoximes are generally more soluble than nitriles in aqueous media. A survey of 975 micro-organisms has shown that aldoxime dehydratase activity is almost invariably linked to nitrile hydrolysis activity.[8] We have adopted vanillin aldoxime **1b** as the standard substrate for screening for nitrilases, because the hydroxyl and methoxy groups confer extra solubility, and at least thus far, aromatic nitriles have been found to be the preferred substrates for most nitrilases.[1]

Using enzymes from thermophilic species in biocatalysis confers considerable benefits.[9,10] The solubility of hydrophobic substrates and rates of diffusion increase as temperature increases and because mesophilic species cannot tolerate high temperatures, it is easier to prevent contamination in whole cell biotransformations. Most thermophilic organisms have been isolated from

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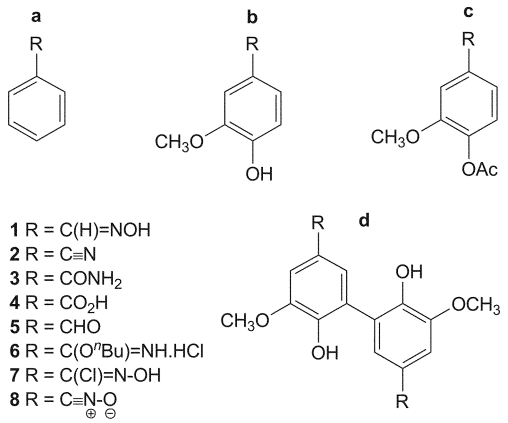
¶ Compounds are numbered using a reverse Markush system. If no alphabetical character is present, the carbon chain is undefined and hence the discussion refers to the generalised chemistry of the functional group indicated.



geothermally heated environments in exotic locations, such as hot springs and deep sea vents, but many of these are now covered by strict licenses for bioprospecting. However under favourable conditions, comparable temperatures are generated by the aerobic decomposition of plant materials in more mundane processes such as composting and ensilaging.

During composting a succession of microorganisms[11] participate in decomposition as the temperature rises to a maximum of 65–82 °C. At the lower end of this range (65–69 °C), *Geobacillus* (previous synonym *Bacillus*) *stearothermophilus* strains (which are obligate thermophiles) frequently predominate,[12,13] and above this temperature (70–75 °C) *Thermus thermophilus* strains (*e.g.* HB8, facultative thermophiles) and several thermophilic *Bacillus* sp.[12,14] are found in high numbers.[15] Although many other species have identified,[16] there is currently much debate over the virtues of different methods (*e.g.* culture, 16S rDNA, chemical markers *etc.*) for determining decomposition-significant microbial populations, rather than those which are amenable to easy identification.[17]

Composting differs from ensilaging by being predominantly aerobic, consequently much of the plant material is lost as carbon dioxide. It is also strongly affected by scale; as the weight of biomass increases, anoxic regions develop at the bottom of the compost pile, which results in the emergence of different microbial populations. Composts also tend to be composed of heterogeneous material at different stages of decomposition. Even in the laboratory, where dog food is the preferred substrate, composting is subject to considerable variation in microbial populations and rates of degradation.[18] In contrast, silage is generally composed of a single plant species, which is harvested at a specific stage of maturity and immediately ensilaged, with controlled water content.

Silage is manufactured from crops such as grass, maize, oats, barley, peas, corn or alfalfa (water content 55–70%), which are compressed (in a silo, clamp or bags) to exclude atmospheric oxygen and allowed to undergo anaerobic fermentation by indigenous, epiphytic microorganisms at 20–35 °C. These are frequently augmented in modern agricultural practice, by inocula of *Lactobacillus*, *Pediococcus* and *Enterococcus* sp.[19,20] to improve reliability and reproducibility. Initially a burst of aerobic decomposition occurs, but when the oxygen in the spaces between the crop is exhausted, anaerobic fermentation commences. In the early stages, sugars are converted to acetic, propionic or butyric acids and in the later stages to lactic acid. The pH drops to around 4, which prevents further degradation, and allows silage to be stored for feeding to cud-chewing animals such as cattle and sheep. If silage is exposed to air[21] then yeast, fungi and bacteria cause aerobic decomposition.[22] If this occurs in bulk silage, (*e.g.* due to leaks in the container), particularly if it too dry (25–45% water) the temperature rises excessively. 70 °C is considered to be an indication of danger, 82 °C the onset of charring and 93 °C* will cause severe losses of material and fire.

We have investigated aerobically decomposing silage as a source of thermophilic micro-organisms with nitrile hydrolysis activity.[23] Surprisingly, silage has rarely been investigated for thermophilic organisms. The first thermophile was isolated by Miquel from the waters of the Seine in 1879, and it was demonstrated that the self heating of hay was due to microorganisms in 1907.[24] Silage was recommended as a good source of thermophiles in 1919,[25] and a review in 1921 reported four previous studies in which thermophilic bacteria growing at 60 °C or above were isolated from silage or hay,[26] but subsequently the area has been largely fallow. Nearly fifty years later, thermophilic "*Bacillus caldotenax*" and "*B. caldovelox*" strains were isolated from silage and used as a source of novel bacteriophages.[27,28] Around the same time *Thermoactinomyces candidus* (synonym *T. vulgaris*) a thermophilic actinomycete (T_{Opt} 50 °C), was first isolated from an air-conditioner duct, but was subsequently found in moldy silage dust. The proteolytic antigens from this species[29] and others[30] are probably one of the causes of hypersensitivity pneumonitis (*e.g.* farmers' lung, humidifier fever). More recently, *Enterococcus faecalis* K-4, was isolated from grass silage in Thailand. Essentially identical cell growth occurred in the range 30–45 °C, but was greatly reduced at 50 °C and maximal amounts of a bacteriocin (an antibacterial peptide) were produced at 43–45 °C.[31] By the usual definitions, both of these species are on the cusp of the temperature range between mesophiles (10–47 °C) and thermophiles (40–68 °C).[32]

Results and discussion

1. Microbiology and biotransformation

Italian rye grass silage samples were incubated on four different media (*Thermus* 878; Difco nutrient broth, Castenholz, Luria-Bertani) at 70 °C in an orbital shaker for 24 hours. Aliquots from enrichment cultures were serially diluted and streaked on *Thermus* 878 agar plates to give 156 colonies with dissimilar colony morphology, which were isolated as pure cultures. Of these, 17 cultures were selected for further investigation on the basis of colony morphological diversity. All the species were Gram positive or Gram variable rods, except for one which was a Gram variable coccid (DDS016) and all produced acid from glucose, fructose and mannose as assessed by reduction in pH. Carbohydrate assimilation profiles (API 50 CHB, bioMérieux, France) and 16S rDNA analysis indicated that all the species could be putatively assigned to the genus *Geobacillus* (formerly *Bacillus* group 5).[33] All were obligate thermophiles (65 °C), except for one (DDS011) which also grew at 37 °C. Sixteen of the isolates were capable of growth as measured by increase in turbidity (OD 600 nm, 24 h) on ammonium chloride or vanillin aldoxime **1b** as sole sources of nitrogen, and one (DDS014) was only capable of slow growth on ammonium chloride. For all the other isolates, vanillin aldoxime

* These seemingly overly precise values are a consequence of conversion from Fahrenheit to Celsius; 180 °F = 82 °C, 200 °F = 93 °C.

1b as sole source of nitrogen caused a significant increase in growth yield ($p < 0.001$) compared to the control (succinic acid), but 35–50% less than ammonium chloride ($p < 0.01$).

The ability of each of the seventeen isolates to transform vanillin aldoxime **1b**, was monitored by HPLC-UV at 254 nm. Traces of hydrolysis products were formed (see below), but the predominant product was a new, unknown compound (later shown to be the dehydro-dimer **1d**) that did not match any of the standards. The degree of transformation was quantified from the UV response of the dehydro-dimer **1d**, relative to that of the initial concentration of vanillin aldoxime **1b** (5 mM). At 24 hours, conversion of vanillin aldoxime to the unknown was a minimum of 11% (DDS017) with the remainder in the range 21 to 54%. At 48 hours, the degree of conversion was evenly distributed in the range 49 to 96% for all seventeen isolates. Even DDS014 which was completely incapable of growth on vanillin aldoxime **1b** alone, nevertheless was capable of forming the dehydro-dimer **1d**, albeit at the lower end of the range observed (24 h, 25%; 48 h 56%). We were not able to repeat the HPLC-UV measurements after the dehydro-dimer **1d** was correctly identified. Moreover, the UV spectra of the dehydro-dimer **1d** in methanol showed a disproportionately increased adsorption at 230–240 nm as the concentration increased, however the maximum which was in the range 260–270 nm (ε *circa* 25 000) was about twice that of vanillin aldoxime **1b** (270 nm, 12 920). Consequently, the percentage conversions reported above are a rough but fair measure of the conversion of vanillin aldoxime **1b**.

Isolate DDS012 had average activity at 24 hours (31%), but had the highest activity at 48 hours and was selected for further investigation. When vanillin aldoxime **1b** was incubated with heat-killed DDS012 cells no transformation occurred. DDS012 grew on vanillin aldoxime **1b** as sole source of nitrogen in the range 1–10 mM, with an optimum of 5 mM (0.0312 doubling h^{-1}). Slow growth occurred in the control which lacked vanillin aldoxime (0.0125 doubling h^{-1}) but 25 or 50 mM vanillin aldoxime inhibited virtually all growth (0.002 doubling h^{-1}). The general bacteriological properties of DDS012 are fairly typical of the group as a whole; it is a Gram positive, small curved rod, with no spores and an obligate thermophile/facultative hyperthermophile. The best species match for carbohydrate assimilation profile (API 50 CHB) was *Bacillus firmus* (ID% 94.4, T-index 0.61) and by 16S rDNA, *Geobacillus thermodenitrificans* OHT-1 (97% sequence similarity, Genbank Accession number EF426762). However the type stain of *G. thermodenitrificans* is able to produce acids from all the sugars tested, but DDS012 was unable to utilise the pentoses: arabinose, ribose, and xylose, the disaccharides: cellobiose and lactose, or galactose and inositol.

As the name of the genus implies, *Geobacillus* is frequently found in soil samples. *G. thermodenitrificans*[34] is closely related to *G. stearothermophilus* which is more commonly isolated from warm composts and comparable niches. The strain *G. thermodenitrificans* OHT-1 has been isolated from horse manure compost under aerobic conditions at 60 °C[35] and *G. thermodenitrificans* CBG-A1, was selected on the basis of L-arabinose isomerase activity at 60 °C from Korean compost.[36]

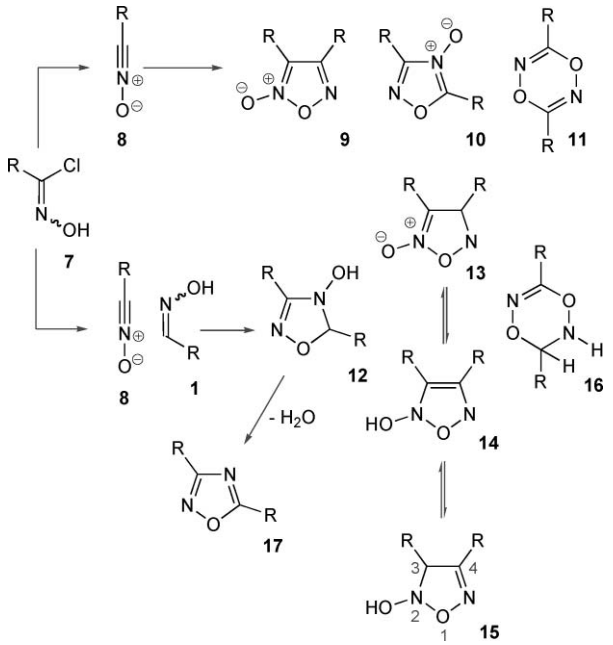
2. Analysis of biotransformation products

The biotransformation of vanillin aldoxime **1b** by DDS012, as described above was reinvestigated in more detail. Up to 7.5 h the rate of transformation of 5 mM vanillin aldoxime **1b**, was 0.35 mM h^{-1} mg^{-1} of dry cells, but thereafter sharply declined to 24 hours and was complete at 48 hours. After 60 hours, no traces of any compounds were detected by HPLC-UV (254 nm). At 24 h, vanillin aldoxime **1b** (5 mM) was converted by isolate DDS012 to a mixture containing traces of vanillin nitrile **2b**, vanillamide **3b**, vanillinic acid **4b** and vanillin **5b**, plus a new component **1d** with a HPLC retention time which did not match any of the standards or conceivable impurities. The vanillin derivatives **2b–5b** were identified by HPLC-QTOF-MS (ESI+) by comparison with commercial samples except for vallinamide **3b**.

There is only one reported synthesis of vallinamide **3b** *via* a five step sequence; in which vanillin **5b** was converted to the aldoxime **1b**, *O*-acetylated and dehydrated to give the 4-*O*-acetyl nitrile **2c**. Cleavage of the *O*-acetyl group gave vanillin nitrile **2b** and electrophilic addition of butanol in hydrogen chloride gave the butyl amidoester hydrochloride salt **6b**, which was finally pyrolysed at 170 °C to give vanillamide **3b** in a reported 95% yield.[37] We repeated this procedure, but found that addition to the nitrile was difficult to achieve in good yield and that the pyrolysis gave no more than a trace of impure product. The obvious synthesis by treating vanillic acid chloride with ammonia is reported to fail,[37] plausibly due to 1,6-elimination of HCl to give a 1,4-quinoketene. We found that a convenient procedure was the DCC mediated coupling of vanillic acid **4b** with *N*-hydroxysuccinimide in DMF. The adduct was soluble whereas most of the DCU precipitated and was removed by filtration. The concentrated solution was then added to concentrated ammonia from which vanillamide **3b** precipitated and was recrystallised to high purity from ethanol in 69% yield.

The predominant component **1d** gave peaks in the mass spectrum at m/z 333 (M + H), 355 (M + Na). Analysis of the accurate mass measurement of the ion at m/z 333 gave seven possible formulae (±10 ppm), from which the best fit and most plausible corresponded to (2 × vanillin aldoxime − 2H), *i.e.* a dehydro-dimer. The functional groups attached to the vanillin ring, could yield many compounds fitting this formula, from reactions such as cycloaddition, peroxide formation or phenolic oxidative coupling. Given that we were seeking new nitrile hydrolysis activity and we were aware of the well precedented dehydration of aldoximes to nitriles by micro-organisms,[6,8] we reasoned that the aldoxime **1b** would undergo dehydration to the nitrile **2b** and oxidation to the nitrile oxide **8b**. Nitrile oxides are well known to undergo cyclodimerisation, which in the current case would give the cycloadducts **9b–11b**, which in turn could be reduced to the dehydro-dimers **12b–16b**. However under abiotic conditions the rate of cyclodimerisation of nitrile oxides is slow and hence the biotic equivalent would require high local concentrations or catalysis of a bimolecular reaction of two highly reactive species. An apparently better alternative was the cycloaddition of nitrile oxide **8b** to the aldoxime **1b** to give dehydro-dimer cycloadducts **12b–16b** directly. Such cycloadditions are unknown in Nature and rare in abiotic synthetic chemistry, but this proposal was more plausible, because it required the reaction of the highly reactive nitrile oxide **8b** with the more stable aldoxime **1b**, which at least in the early stages of the process would be present in high excess.

Aromatic nitrile oxides **8** most commonly cyclodimerise to give 3,4-diaryl-1,2,5-oxadiazol-2-oxides **9**, but 3,5-diaryl-1,2,4-oxadiazol-4-oxides **10** are occasionally found as byproducts.



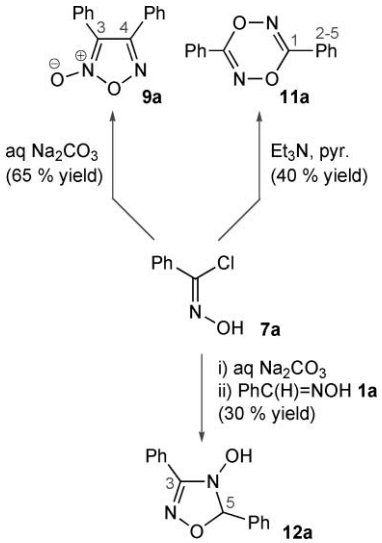
Whereas in the presence of nucleophiles (*e.g.* pyridine[38,39] or trimethylamine[40]), addition, cycloaddition, elimination gives 3,6-diaryl-[1,4,2,5]dioxadiazines **11** or tricyclic derivatives.[41] To further increase the chemical diversity; dimerisation of benzonitrile oxide **8a** in the presence of excess boron trifluoride gives 3,6-diphenyl-[1,4,2,5]dioxadiazines **11a**, but with only half an equivalent of BF_3; 3,5-diaryl-1,2,4-oxadiazol-4-oxides **10a**,[42,43] whereas with boron trifluoride etherate, both of these products are formed plus the isocyanate.[44]

As far as we are aware there has only been one report of the cycloaddition of a nitrile oxide to oximes. Benzonitrile oxide **8a** underwent addition to two aldoximes and two ketoximes catalysed by boron trifluoride etherate to give 4-hydroxy-4,5-dihydro-1,2,4-oxadiazoles **12**,[45] although clearly there are other possibilities **13**–**16**. Of these latter four ring systems, the sole isolated example is 3-chloro-3,4-diphenyl-3*H*-furazan-2-ol[46] (*cf.* **15**), although another example may be a minor component of a tautomeric mixture.[47]

Most of this work was reported before the advent of high field NMR and some structures for the adducts have been controversial.[48,49] Faced with this melange of possible structures and a paucity of reliable NMR data, we took prudent refuge on the safer ground of initial model studies.

3. Cycloaddition of phenyl derivatives

Chlorination of (*Z*)-benzaldoxime **1a**[50] with *N*-chlorosuccimide in THF yielded the oximyl chloride **7a** (82% yield), which could be stored in the freezer for several weeks without decomposition. Treatment of the oximyl chloride **7a** with aqueous sodium bicarbonate gave 3,4-diphenyl-1,2,5-oxadiazol-2-oxide **9a**, whereas treatment with triethylamine and pyridine gave 3,6-diphenyl-[1,4,2,5]-dioxazine **11a** as reported previously.[38] The ^{13}C-NMR data for 3,4-diphenyl-1,2,5-oxadiazol-2-oxide **9a** in DMSO-d_6 was identical to partial data reported previously (δ_C 114.5, 3-C; δ_C 156.6, 4-C).[51] Virtually nothing could be deduced from the ^{1}H-NMR spectrum, because of overlap, however in deuterated chloroform, virtually all the signals could be assigned. The key NMR data for 3,6-diphenyl-[1,4,2,5]-dioxazine **11a** (δ_C 162.7, 1-C; 125.6, 2-C) were reassuringly close to that for 3,6-di(2-pyridyl)-1,4,2,5-dioxadiazine (δ_C 160.63, 1-C)[52] and more relevantly for 3,6-di(4-tolyl)-1,4,2,5-dioxadiazine (δ_C 162.8, 1-C), although only this shift was reported with no supporting data.[53]

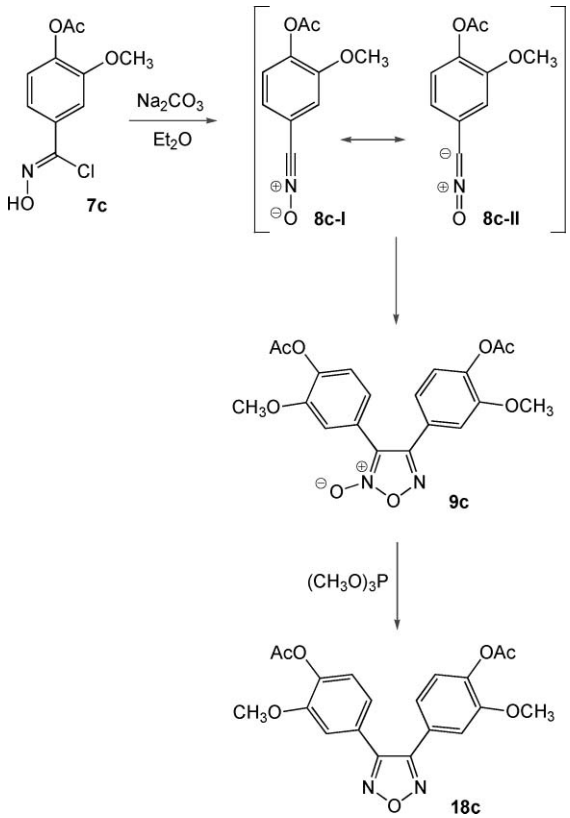


To give some assurance that we were truly preparing the nitrile oxide-aldoxime adduct, a split experiment was devised. Treatment of oximyl chloride **7a** with aqueous sodium bicarbonate as before, gave a diethyl ether solution of nitrile oxide **8a** which was divided into two. One portion was allowed to stand at room temperature and gave 3,4-diphenyl-1,2,5-oxadiazol-2-oxide **9a** as the sole product after 20 h by ^{1}H-NMR spectrometry and was isolated in 58% yield. The other portion was added to (*Z*)-benzaldoxime **1a** but otherwise treated in an identical way. The crude reaction mixture containing the nitrile oxide–aldoxime adduct **12a**, dimer **9a** and other products was separated by column chromatography. Crystallisation yielded 3,5-diphenyl[1,2,4]oxadiazol-4-ol **12a** (32%), which was unstable in deuterated chloroform. However the adduct was perfectly stable in deuterated benzene and the NMR spectra were fully assignable. The NMR chemical shifts for 5-C/H (δ_H 6.08, δ_C 101.7, C_6D_6) were poorly predicted by chemical shift increment models (δ_H 5.64, δ_C 84.1, $CDCl_3$) even allowing for the solvent difference. The key signal validating the assignment was a ^{13}C–^{1}H–^3J-correlation signal between 5-H and 3-C (δ_C 159.2; C_6D_6). The mass spectrum (EI+) showed no molecular ion, but a strong M–H_2O peak, consistent with *in situ* dehydration to 3,3-diphenyl-1,2,4-oxadiazole **17a**.[54]

4. Cycloaddition of vanillin derivatives

4-*O*-Acetyl vanillyl nitrile oxide **8c** proved to be very difficult to handle. After considerable experimentation we found that addition of the oximyl chloride **7c** to a vigorously stirred sodium carbonate solution, rapid work-up and dissolution in deuterated benzene allowed us to obtain NMR spectra of the nitrile oxide **8c** which slowly dimerised to the dimer **9c** at ambient temperature. In the first 12 h we were able to detect the ^{13}C-NMR signal for the nitrile oxide carbon at δ_C 34.4, which appeared as a very broad triplet with peak heights in the ratio 1 : 1.6 : 1 and a coupling of *circa* 50 Hz ($^1J^{13}$C,^{14}N). Coupling to ^{14}N should yield three equally intense, but broad lines due to ^{14}N nuclear quadrupole

relaxation, however the extreme line broadening causes the height of the central line to be increased by overlap with the outer lines. Our results are comparable to those of reported for ^{15}N-2,4,6-trimethylbenzo- (δ_C 34.9,[55] d, ^1J-^{13}C,^{15}N 77.5 ± 0.5 Hz, in CD_2Cl_2[56]), 2,4,6-trinitrobenzo- (δ_C 28.93[57]), aceto- (δ_C 35.6, br t, −51 °C[58]) *tert*-butylaceto- (δ_C 42.2, br t, > 50 Hz[59]) and triphenylmethylaceto-(δ_C 38.4, br[59]) nitrile oxides in $CDCl_3$. The chemical shift of the "nitrile oxide" carbon is best rationalised as the iminyl carbanion canonical form **8c**–**II** rather than the nitrile oxide **8c**–**I**.



ortho-Disubstituted nitrile oxides are frequently sufficiently stable that they can be isolated and analysed, however the *case in hand* lacks this steric barrier which tempted us to determine the rate constant for dimerisation. The method of preparation does not allow an accurate measure of the start time, consequently this was estimated and used as an additional data point. Fortuitously, the single *O*-methyl group of the nitrile oxide was easily distinguishable from the two of the dimer by ^{1}H-NMR and there was no overlap with other signals, consequently integration of these signals enabled a relatively accurate measure of the composition of the reaction mixture. Six data points were measured over a molar composition range; 84 : 16 to 15 : 85 for nitrile oxide **8c** : dimer **9c** over 60.75 h, which were analysed by plotting 1/[nitrile oxide] *vs.* time, with the assumption of second order dimerisation kinetics (Fig. 1). The rate constant from linear regression was 0.434 M^{-1} h^{-1} or 1.21 × 10^{-4} M^{-1} s^{-1} (R^2 = 0.976) for a solution which was initially 0.413 M (95% C_6D_6, 5% THF *wt*/*wt*). The initial half life was calculated to be 5.6 h, and the curve fit to the data indicated a value around 4.8 h (for 66 : 33, **8c** : **9c**). In the initial stages the conversion is very rapid and the effect of the poorly estimated starting time has maximum effect. After a total of 380.07 h (15.8 days) the reaction mixture consisted almost entirely of dimer **9c**, plus traces of unidentified impurities (*circa* 2%) and nitrile oxide **8c**. Ignoring the impurities, the ratio of nitrile oxide **8c** to dimer **9c** was 2.8 : 97.2, which corresponds to 5.86 mM nitrile oxide **8c**. Remarkably, the predicted value from the rate equation was 5.93 mM an error of just 1.2%. In the sole prior report of the kinetics of dimerisation of nitrile oxides, rate constants were measured for benzonitrile oxide and four monosubstituted derivatives, Values ranged from 2.37 (*p*-methoxy) through 6.78 (*p*-chloro) to 8.53 (*m*-chloro) × 10^{-3} M^{-1} s^{-1} at 40 °C in carbon tetrachloride, which is appreciably higher than the value we measured (0.121 × 10^{-3} M^{-1} s^{-1} at *circa* 25 °C), even allowing for the temperature difference. At 25 °C the rate constant for the dimerisation of *p*-chlorobenzonitrile oxide in carbon tetrachloride was 1.81 × 10^{-3} M^{-1} s^{-1} but in chloroform only 0.177 × 10^{-3} M^{-1} s^{-1}. Hence dimerisation rates in carbon tetrachloride are up to ten times faster than in other solvents.[60] No data was reported for benzene or even a comparable solvent, so no sensible comparison can be made between the two sets of data. However by using the data for *p*-chlorobenzonitrile oxide in chloroform at 25 °C and at 40 °C in carbon tetrachloride, the rate constant for the dimerisation of 4-*O*-acetyl-vanillin nitrile oxide in carbon tetrachloride at 40 °C is estimated to be *circa* 4.6 × 10^{-3} M^{-1} s^{-1} which is in satisfactory agreement with the prior data. The structure of the dimer **9c** was determined from NMR spectra and by reduction with neat trimethylphosphite at reflux to yield the symmetrical product; 3,4-diaryl-1,2,5-oxadiazole **18c**.

The cycloaddition of 4-*O*-acetyl vanillyl nitrile oxide **8c** with 4-*O*-acetyl vanillin aldoxime **1c** proved to be very difficult and despite testing a range of reaction conditions, only barely acceptable results were finally achieved. Initially, 4-*O*-acetyl vanillyl oximyl chloride **7c** was treated with saturated sodium carbonate in diethyl ether. Workup gave a solution of nitrile oxide **8c** which was added to a 1 : 1 mixture of 4-*O*-acetyl vanillin aldoxime **1c** and boron trifluoride etherate according to the literature method.[45] An aliquot of the nitrile oxide solution showed a 75 : 25 mixture of nitrile oxide **8c** to dimer **9c** by ^{1}H-NMR. This measurement indicates that the solution contained essentially pure nitrile oxide with little or no dimer, because the time taken to prepare and run the NMR spectrum, allows time for dimerisation. After two hours the reaction consisted of a 50 : 50 mixture of nitrile oxide **8c** and the aldoxime **1c**, which became a 23 : 77 mixture of dimer **9c** and aldoxime **1c** after 60 hours. In a control reaction a 1 : 1 mixture of 4-*O*-acetyl vanillin aldoxime **1c** and boron trifluoride etherate in diethyl ether was stirred for 48 hours and the aldoxime **1c** was recovered in quantitative yield. Reverting to the conditions used for the phenyl nitrile oxide **8a** and benzaldoxime **1a** were equally unrewarding. The oximyl chloride **7c** was treated with sodium carbonate in THF and the nitrile oxide solution added to 1.5 equivalents of aldoxime **1c** in dichloromethane. ^{1}H-NMR monitoring of aliquots showed a 21 : 79 mixture of nitrile oxide **8c** : aldoxime **1c** after 2.5 hours and a 13 : 87 ratio after 23.5 hours and after workup a 7 : 93 ratio. Curiously, no dimer **9c** was observed at any stage in this reaction. In an attempt to mimic the conditions of the successful dimerisation of nitrile oxide **8c**, essentially the same reaction was conducted in deuterated benzene. After 2 hours a ^{1}H-NMR spectrum of an aliquot showed a 9 : 13 : 78 ratio of nitrile oxide **8c** : dimer **9c** : aldoxime **1c**, which became an 18 : 82 ratio of dimer **9c** : aldoxime **1c** after 17 hours. Preparation of scrupulously

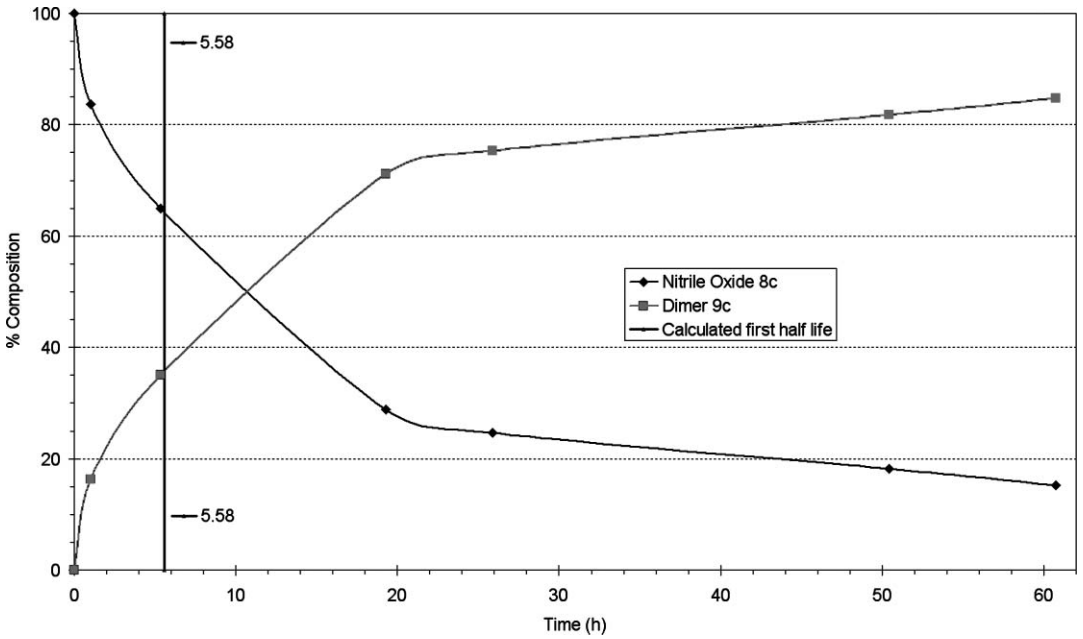
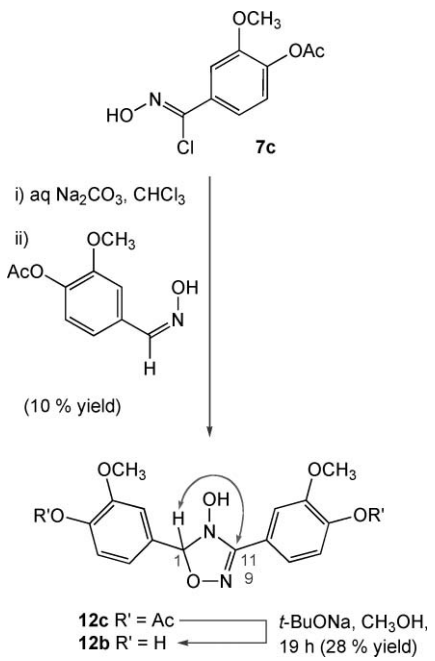


Fig. 1 Nitrile oxide and dimer conversion, % units.

dry nitrile oxide from ethereal solutions, whilst working quickly to avoid dimerisation is difficult. Consequently, we decided to prepare the nitrile oxide **8c** and react it with the aldoxime **1c** in chloroform, which is easier to dry. The crude ^{1}H-NMR revealed a new down field singlet and column chromatography gave the desired adduct **12c** in 10% yield. The connectivity of the heterocyclic ring was established from NMR spectra in which C-1, δ_C 101.1 was attached to a hydrogen at δ_H 6.07 with a ^{3}J-^{13}C–^{1}H coupling to a carbon at δ_C 159.2 (C-11). Deacetylation with sodium *tert*-butoxide in methanol gave the imagined natural product **12b** in a miserable 28% yield after column chromatography. The NMR spectroscopic data for C-1/H-1 and C-9 (C_6D_6 δ_H 6.23; δ_C 101.7; 159.6) were essentially identical to that of the acetylated precursor and we also obtained a second set of NMR data in DMSO-d_6 to make absolutely certain that the structure was rigorously assigned. When this material was analysed by HPLC-QTOF-MS (ESI+), the retention time was identical to that of the natural product, but the mass spectrum was quite different. The natural product **1d** showed a high abundance precursor ion (M + H, *m/z* 333.1) plus a series of product ions of diminishing abundance down to *m/z* 212.1, due to small losses (H_2O, CH_3OH *etc.*), whereas the supposed natural product, showed an almost negligible precursor ion, two high abundance product ions at *m/z* 168.1 (plausibly protonated vanillin aldoxime **1b** + H^+) and 135.1 and some weaker ions in between (Fig. 2).



5. Phenolic oxidative coupling

At this stage we reassessed the mass spectrum data for the natural dehydro-dimer and the circumstantial evidence surrounding its formation. Firstly, the mass spectrum of the heterocyclic dehydro-dimer **12b** clearly showed cleavage of the heterocyclic ring yet the mass spectrum of the natural product **1d** showed only progressive losses of small fragments, consistent with a single carbon chain. Secondly the natural dehydro-dimer was only formed with vanillin aldoxime **1b** and no analogous compound was formed from 2,3-dimethoxy- or 2,4-dimethoxybenzaldoxime indicating that the free phenol substituent of vanillin aldoxime was crucial for dimerisation formation. Finally, formation of the dimer was always attended by formation of a yellow pigment, with an apparently high ε-value, because the colour was strong even at high dilution. Again this could not be isolated, but the yellow colour was reminiscent of quinones formed by an oxidative process. Taken together, this admittedly tenuous evidence suggested that the dehydro-dimer was the product of phenolic oxidative coupling.

Phenol oxidative coupling of vanillin **5c** is a well known process and indeed dehydro-divanillin **5d** is a commercial product. It was first prepared in 1916 using potassium persulfate and

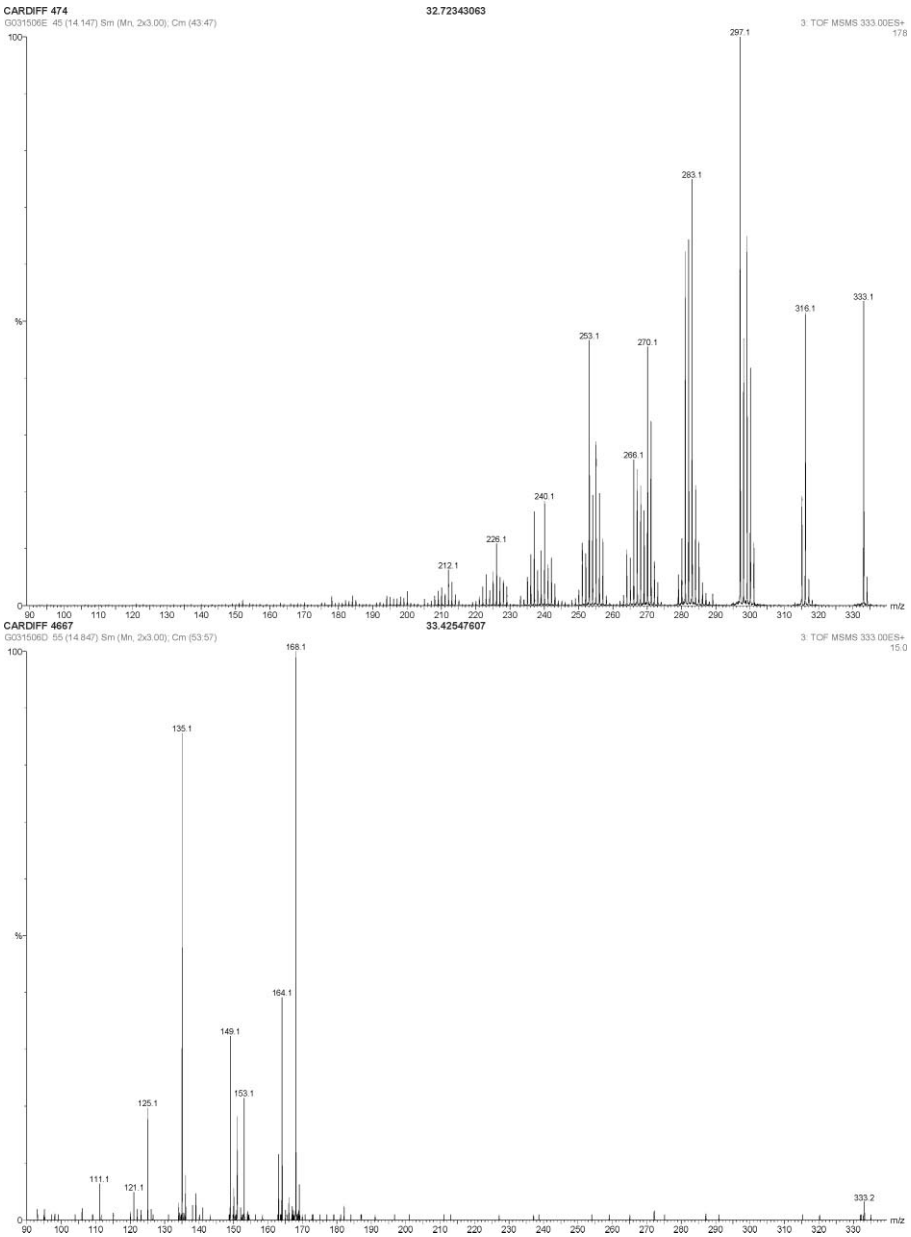


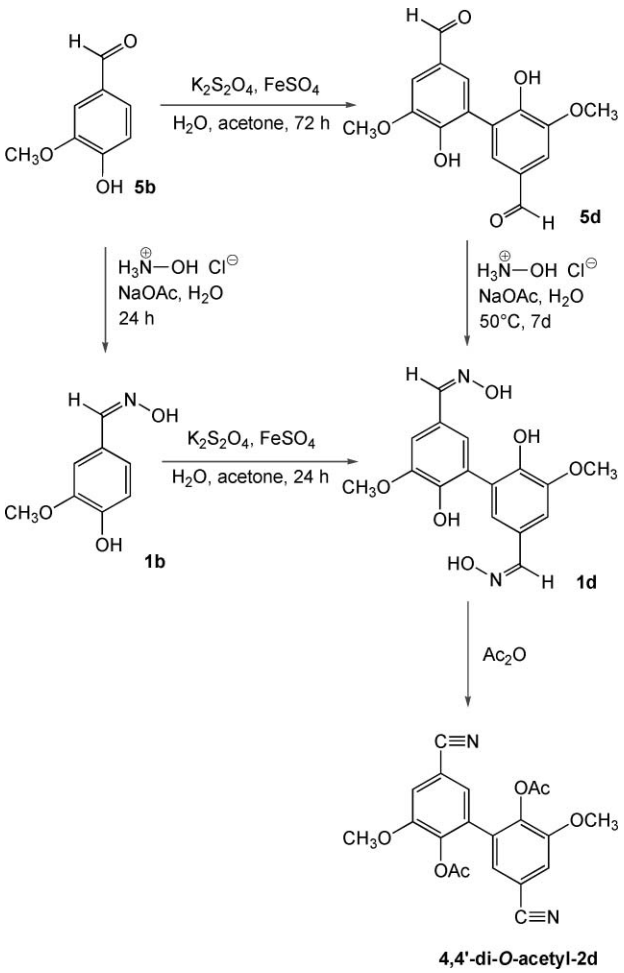
Fig. 2 HPLC-QTOF-MS/MS (ESI+) spectra of vanillin aldoxime dehydro-dimer **1d** (top) and the imagined-natural product, 3,5-di(4-hydroxy-3-methoxyphenyl)-5*H*-[1,2,4]oxadiazol-4-ol **12b** (bottom).

catalytic ferrous sulfate in almost quantitative yield (97%[61]), but repetition of this procedure has been less fruitful (22%[62] & 86%[63]). Other procedures have used ferric chloride·hexahydrate (57%[64]), TEMPO derived oxoammonium salts (85%[65]) or [(diacetoxy)iodo]benzene (56%[66]). The kinetics of octacyanotungstate(v) ion mediated oxidation in alkaline solution (80%) are first order in vanillin anion and $[W(CN)_8]^{3-}$.[67] It has also been prepared by the Ullman coupling of 5-bromovanillin with copper powder in anhydrous DMF, but no yield was reported.[68] Biotransformation based methods are discussed below.

For our own satisfaction we performed the phenolic oxidative coupling of vanillin with; potassium persulfate and iron sulfate[61] and achieved a moderate yield of dehydrodivanillin **5d** (47%, identical to commercial material), plus recovered vanillin **1d** (23%). Formation of the dialdoxime **1d** was extremely slow, using hydroxylamine and sodium acetate. Nevertheless after 7 days at 50 °C, 40% conversion was determined by ^{1}H-NMR and the dehydro-dimer **1d** was isolated (33% yield). Alternatively treatment of vanillin aldoxime **1b** with potassium persulfate and catalytic ferrous sulfate gave a good yield of crude product **1d** (71%), which was hugely eroded by washing/crystallisation to give material of high purity (19%). The dehydodimer **1d** produced by each route was identical and identical to the natural product by HPLC-MS, on the basis of retention time, accurate mass measurement and mass spectral fragmentation patterns.

One of the reasons that we initially discounted phenolic oxidative coupling as a route to a dehydro-dimer **1d** was that we were unable to find an example of this reaction for aryl

oximes in the literature. It appeared reasonable that the aldoxime would be become oxidised either to a nitro-compound or some intermediate oxidation state. However careful reading revealed that Erdtman had reported that the dehydro-dimer **1d** could be prepared by potassium persulfate and iron sulfate coupling of vanillin aldoxime **1b**. The product was not isolated (and hence does not appear in the databases) but was immediately acetylated and dehydrated to the acetylated nitrile 4,4′-di-*O*-acetyl-**2d**.[69] This appears to be the only reported case of phenolic oxidative coupling of an aryl oxime. However in recent syntheses of spiroisoxazole sponge metabolites, homobenzylic aldoximes have been used to capture phenolic radicals and thereby yield spiro-1,2-isoxazolinyl-monoquinones.[70]



The abiotic preparative work described here clearly shows that vanillin **5b** and vanillin aldoxime **1b** react in comparable ways when subjected to the standard conditions for phenolic oxidative coupling. Vanillin **5b** is easily oxidised to dehydro-divanillin **5d** by hydrogen peroxide catalysed by soybean peroxidase (1–15%),[71] or in better yield by horseradish peroxidase, (97%,[72] 88%[73]), even in UHT milk![74] Dehydro-divanillin **5d** has been frequently used as a lignin mimic[75] and indeed it is a product of the action of the wood degrading enzyme; laccase on 3,3′-dimethoxy-5,5′-dimethyl-biphenyl-2,2′-diol (0.1–0.2%).[76] Dehydro-divanillin **5d** is cleaved to vanillin **5b** by the anaerobic recombinant FE7 which was created by protoplast fusion of *Fusobacterium varium* and *Enterococcusfaecium*[77] and dehydro-divanillinic acid **4d** to 5-carboxyvanillate by the soil bacterium *Pseudomonas paucimobilis* SYK-6.[78,79]

16S rDNA data indicated that isolate DDS012 was related to *Geobacillus thermodenitrificans* OHT-1. The closest related species for which full genetic data is available is *G. stearothermophilus*. This was originally reported to express a novel peroxidase from the *perA* gene,[80] but after sequencing errors in the gene were corrected, it is now termed Catalase I.[81] This is a member of Class I of the superfamily of heme containing plant, fungal and bacterial peroxidases, which consists of bacterial catalases with broad spectrum peroxidase activity. Despite high catalase activity, they have minimal sequence homology with typical heme containing monofunctional catalases, but have high sequence homology to plant ascorbate peroxidase and fungal cytochrome *c* peroxidase.[82] Catalase I has the interesting property that upon heating to 70 °C for ten minutes it undergoes a conformation change to a form with higher catalase activity, which only reverts to the lower temperature form after denaturation, refolding and reconstitution with heme.[83] Clearly, Catalase I is an ideal candidate for catalysing the oxidative dimerisation of vanillin aldoxime **1b** at high temperatures. Perforce the benefit of this process to the organism is open to many interpretations, but we imagine that conversion to the highly insoluble dehydro-dimer **1d** may constitute detoxification by sequestration. Moreover consumption of peroxide is beneficial to all aerobic organisms, but particularly for thermophiles.

Acknowledgements

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