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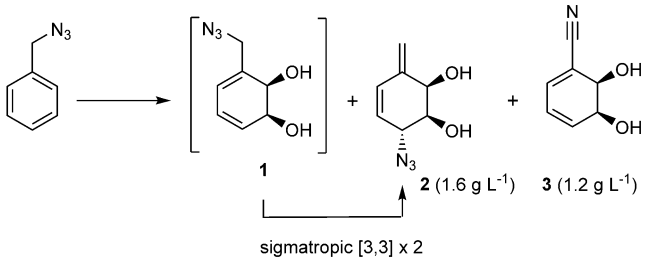
Toluene Dioxygenase-Catalysed Oxidation of Benzyl Azide to Benzonitrile: Mechanistic Insights for an Unprecedented Enzymatic Transformation

María Agustina Vila[+],[a] Mariana Pazos[+],[a] César Iglesias,[b] Nicolás Veiga,[c] Gustavo Seoane,[a] and Ignacio Carrera*[a]

Enzymatic dioxygenation of benzyl azide by toluene dioxygenase (TDO) produces significant amounts of the *cis*-cyclohexadienediol derived from benzonitrile, along with the expected azido diols. We demonstrate that TDO catalyses the oxidation of benzyl azide to benzonitrile, which is further dioxygenated to produce the observed *cis*-diol. A proposed mechanism for this transformation involves initial benzylic monooxygenation followed by a nitrene-mediated rearrangement to form an oxime, which is further dehydrated to afford the nitrile. To the best of our knowledge, this is the first report of enzymatic oxidation of an alkyl azide to a nitrile. In addition, the described oxime-dehydration activity has not been reported for Rieske dioxygenases.

Research on new methodologies for the preparation of nitriles is of high interest to the organic chemistry community, because they are considered very useful building blocks for the preparation of pharmaceuticals and functional material.[1] In recent years, the oxidative transformation of alkyl azides into nitriles has attracted much attention; it is an interesting approach for the synthesis of nitriles because it does not require elongation of the skeletal carbon chain. Several methods for this transformation have used stoichiometric oxidant, with or without a catalyst, for example, BrF_3,[2] bis(acetoxy)iodobenzene,[3] 2,3-dichloro-5,6-dicyanobenzoquinone,[4] *tert*-butylhydroperoxide,[5] $Pd(OAc)_2$,[6] $Ru(OH)x/Al_2O_3$[7] and electrochemical anodic oxidation.[8]

Recently, we reported the structures of novel interesting azido diols (Scheme 1) obtained from the biotransformation of



Scheme 1. Biotransformation of benzyl azide by *E. coli* JM109 (pDTG601) in a 5 L bioreactor by the previously described bi-phasic protocol.

benzyl azide by the toluene dioxygenase (TDO) enzymatic complex from *Pseudomonas putida* F1 expressed in *Escherichia coli* JM109 (pDTG601).[9] In addition to the expected diol **1**, the exocyclic diene **2** was found; its formation was explained by an spontaneous stereoselective double sigmatropic [3,3] shift from **1**. Significant amounts of *cis*-cyclohexadienediol, **3,** were obtained as a third product, derived from benzonitrile.

Here we demonstrate that TDO catalyses the oxidation of benzyl azide to benzonitrile, which is further dioxygenated to produce the observed *cis*-diol **3**. To the best of our knowledge, this is the first report of enzyme-catalysed oxidation of a benzylic azide to a nitrile. Although it is well known that bacterial dioxygenases are able to catalyse oxidations other than the widely described dihydroxylation of aromatic rings (e.g., monooxygenation of benzylic and allylic C–H bonds, sulfoxidations, O- and N-dealkylations and oxidative carbocyclisations),[10,11] the described transformation cannot easily be explained by these activities. A plausible mechanism is presented.

The formation of nitrile **3** involves two processes: oxidation of the primary azide functionality and dioxygenation of the aromatic ring by TDO. This chained oxidation can occur from either the benzylic azide in the starting material or from the allyl azide in the *cis*-cyclohexadienediol **2**, and can be chemically or enzymatically mediated (by the TDO complex or another enzyme in the biocatalyst). In order to answer these questions we carried out the biotransformation of **1** in resting cells of *E. coli* JM109 (pDTG601) (Figure 1 A); only diol **2** was detected as a product, formed by spontaneous two [3,3] sigmatropic shifts, as previously described.[9] Because **3** cannot be produced from **1**, benzonitrile (a known substrate for TDO)[12] should be formed as a first intermediate prior to dioxygenation. We carried out a control experiment with *E. coli* JM109 (lacking the genes for TDO) to assess other enzymes in nitrile generation (Figure 1 A). Benzonitrile was not detected, and

[a] *M. A. Vila,[+] M. Pazos,[+] Dr. G. Seoane, Dr. I. Carrera*
Departamento de Química Orgánica
Facultad de Química, Universidad de la República
Av. General Flores 2124, C.P. 11800 Montevideo (Uruguay)
E-mail: icarrera@fq.edu.uy
Homepage: http://www.secobi.fq.edu.uy/

[b] *C. Iglesias*
Cátedra de Microbiología, Departamento de Biociencias
Facultad de Química, Universidad de la República
Av. General Flores 2124, C.P. 11800 Montevideo (Uruguay)

[c] *Dr. N. Veiga*
Cátedra de Química Inorgánica, Departamento Estrella Campos
Facultad de Química, Universidad de la República.
Av. General Flores 2124, C.P. 11800 Montevideo (Uruguay)

[+] *These authors contributed equally to this work.*

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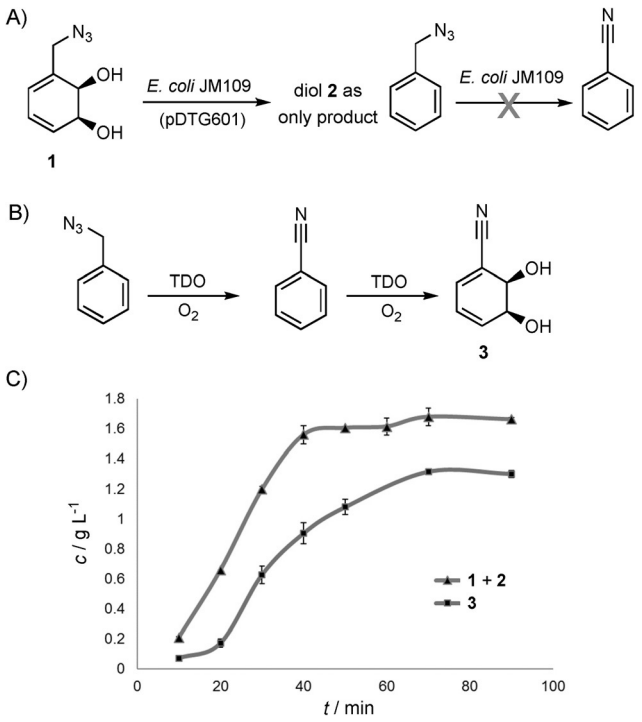
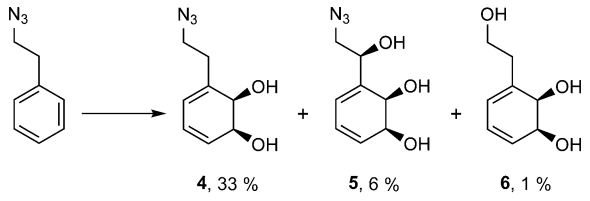


Figure 1. A) Diol **1** is not a substrate for TDO. *E. coli* JM109 does not oxidize benzyl azide to benzonitrile. B) Proposed sequence for the generation of diol **3**. C) HPLC product formation profiles for benzyl azide biotransformation in a 5 L bioreactor by using a biphasic protocol. Formations of diols **1**+**2** are expressed together to simplify data analysis.

only unreacted benzyl azide was recovered. This strongly suggested the participation of the TDO complex in the oxidation. In the proposed formation of **3** (Figure 1 B), TDO catalyses the initial oxidation to form benzonitrile, which is subsequently dihydroxylated by the same enzymatic complex. We next studied product formation profiles in a biotransformation carried out in a 5 L bioreactor (Figure 1 C).[13] The plots show an initial lag phase for the formation of cyanodiol **3**, and then both curves (which represent the formation of products **1**+**2** and formation of cyanodiol **3**) display comparable initial slopes. These profiles are consistent with the proposed initial formation of benzonitrile from benzyl azide and subsequent dioxygenation to **3**.

In order to deepen our understanding of the benzyl azide oxidation to benzonitrile by the TDO complex we studied the biotransformation of phenylethylazide (Scheme 2). Three *cis*-cyclohexadienediols (**4**, **5** and **6**) were isolated as the only products (overall yield, 40 %) along with unreacted starting material. To our surprise, no nitrile functionality was found in any product. The expected diol **4** accounted for 80 % of the products, but the formation of triols **5** and **6** deserves some comment. The formation of **5** could be explained by the known benzylic monooxygenation activity of TDO followed by ring dioxygenation.[10e] Formation of **6** is intriguing, given that it was not a result of azide displacement by water, as phenethylic alcohol was not detected in the *E. coli* JM109 control; this is explained below.



Scheme 2. Biotransformation of phenylethylazide by *E. coli* JM109 (pDTG601) in resting cells.

It was evident that nitrile formation by the TDO complex requires the azide functionality to be placed at the benzylic position. Given the formation of **5**, for the case of benzyl azide we hypothesized that benzylic monooxygenation could occur as the first step in the production of benzonitrile. Benzylic C–H oxygenation by TDO has been extensively studied for alkyl arenes.[10e, 14] Usually benzene rings that contain a benzylic C–H bond can be monooxygenated by TDO to give alcohols; these are better substrates than their alkyl precursors, and thus undergo rapid *cis*-dihydroxylation to yield enantiopure triols. This monooxygenation activity is highly sensitive to the arene substitution pattern; for example 1,4-disubstituted arenes are not good substrates for benzylic monooxygenation by TDO.[10e]

In order to obtain evidence that initial monooxygenation at the benzylic position mediates nitrile formation by TDO, *E. coli* JM109 (pDTG601) was supplied with *p*-chlorobenzyl azide as a substrate (Figure 2 A). Nitrile formation was completely abolished: diol **7** was the only product found in the reaction, along with unreacted starting material. Also, no azide-rearranged product was found.

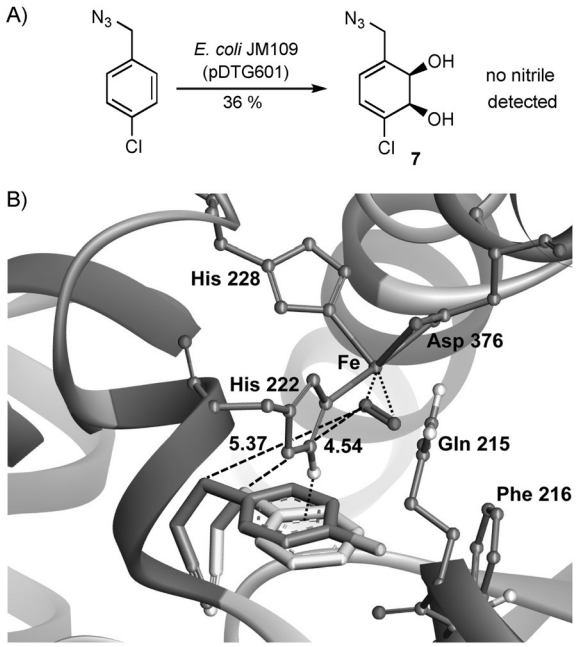
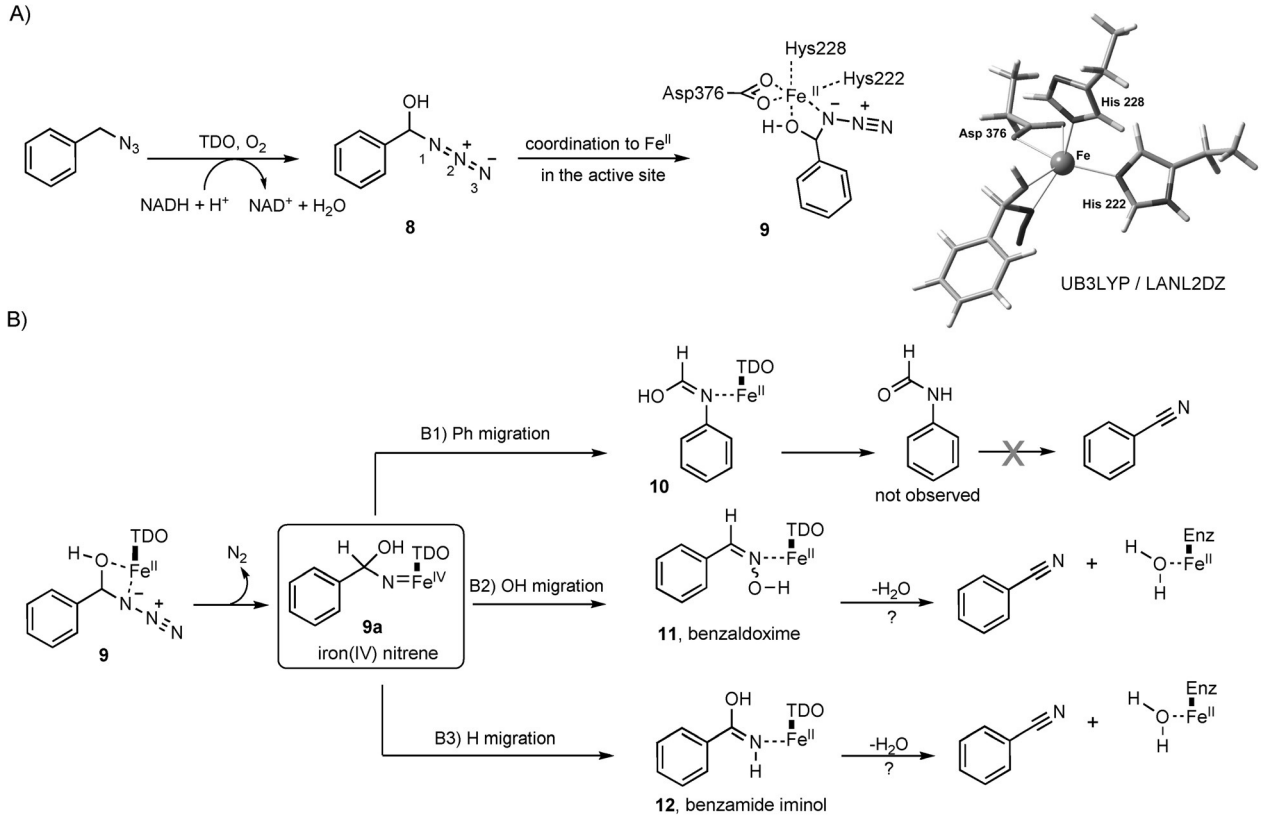


Figure 2. A) Nitrile formation activity was abolished when *para* substitution was made in the aromatic ring. B) Model of *p*-chlorobenzyl azide and benzyl azide (yellow) docked in the active site of TDO (PDB ID: 3EN1): C (grey), N (blue), O (red), H (white), Cl (green).

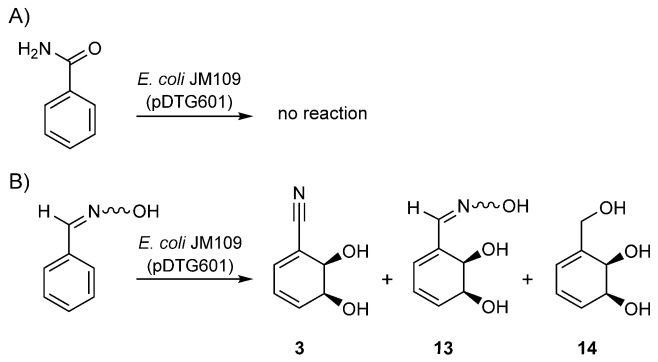
Based on the X-ray structure of TDO,[15] we performed molecular docking to rationalise this result. *p*-Chlorobenzyl azide and benzyl azide were docked into the active site of TDO (Figure 2B): residues Gln215 and Phe216 form a "wall" at the active site, thus preventing lateral movement of the substrates. As a consequence, whereas the benzylic position of the monosubstituted substrate is 4.54 Å from the dioxygen molecule bound to the Fe^{II} ion, for the 1,4-disubstituted substrate this distance is greater (5.37 Å, which prevents benzylic monooxygenation from taking place). The results are in full agreement with the prediction of Boyd et al. based on the similarities of the active sites of TDO and NDO.[10f]

Thus, both the formation of **5** from phenylethylazide and the lack of nitrile formation in *p*-chlorobenzyl azide suggest that initial monooxygenation at the benzylic position to yield the α-hydroxyl azide **8** mediates nitrile formation. Scheme 3 shows a possible route for the overall oxidation process from **8**. According to computational results at the UB3LYP/LANL2DZ level of theory, coordination of **8** to Fe^{II} in the TDO active site would be highly favoured. By using the metal and amino acid coordinates of the TDO crystal structure, we obtained the most stable geometry for the resulting coordination complex **9** (Scheme 3A). The structural constraints imposed by the protein architecture lead to the formation of a high-spin adduct, for which the resulting four-membered chelate ring is stable enough to support bidentate coordination through the hydroxylic oxygen and nitrogen N1 of the azide. Azides can bind transition metals by the carbon-attached nitrogen (N1) or by coordination with the terminal nitrogen (N3).[16] N1 coordination results in a shortening of the terminal N2–N3 bond and a weakening (elongation) of the bond between N1 and N2, thereby facilitating loss of dinitrogen (N_2).[16] For **9** the calculated Wiberg bond indexes (bond orders) are 1.45 (N1–N2 bond) and 2.34 (N2–N3), thus nitrogen loss could be favoured thereby resulting in a iron(IV)–nitrene intermediate **9a** (Scheme 3B). The generation of an imido–iron(IV) complex in iron-containing monooxygenases (equivalent to **9a**) has been recently described for acyl azides in the P450-catalysed conversion of carbonazidates to oxazolidinones.[17]

Given the high reactivity of iron(IV)–nitrene species, rearrangement of the nitrene to provide a more stable Fe^{II}-coordinated compound is proposed (Scheme 3B). Migration of the phenyl group (pathway B1) would afford **10**, which would render the corresponding formylaniline or its dioxygenated derivative (not found in the reaction medium). In the case of OH or H group migration, the corresponding benzaldoxime **11** or benzamide's iminol **12** would be obtained (pathways B2 and B3, respectively). Both products could be dehydrated to give benzonitrile, thus implying either oxime or nitrile dehydratase activity by TDO (or other enzyme present in the microorganism), as spontaneous dehydration would not be expected in the reaction medium. In order determine the correct pathway we used benzaldoxime (*E* and *Z* isomers) and benzamide as substrates with *E. coli* JM109 (pDTG601). Benzamide gave no

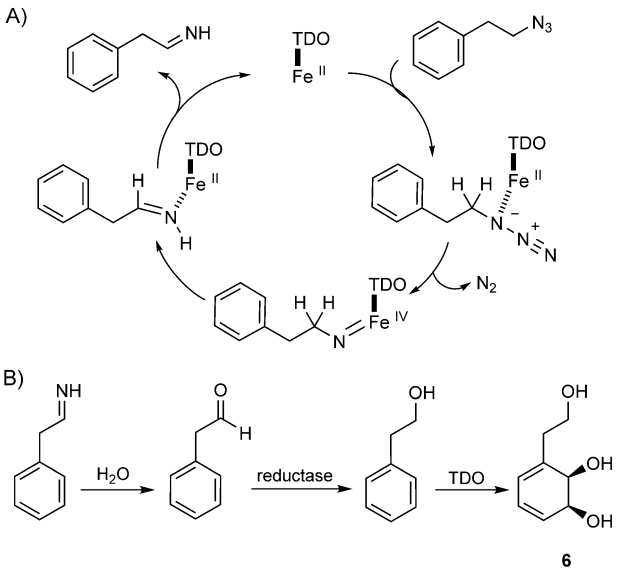


Scheme 3. A) Monooxygenation of benzyl azide by TDO gives hydroxyazide **8**, which could coordinate to Fe^{II} in the active site of the enzyme. This bidentate coordination is supported by computational calculations at the UB3LYP/LANL2DZ level of theory. B) The proposed coordination could promote nitrogen loss to form an iron(IV)–nitrene complex, which could rearrange via three pathways (B1, B2 and B3).



Oxime	Yield / %	Ratio **3** / **13** / **14**
Z	12	33 : 25 : 42
E	10	20 : 20 : 60

Scheme 4. A) Benzamide is not a substrate for TDO. B) Biotransformation of *Z* and *E* benzaldoximes by *E. coli* JM109 (pDTG601).



Scheme 5. Proposed mechanism for the formation of **6**.

reaction (all starting material recovered; Scheme 4A). However, *Z* and *E* benzaldoximes afforded nitrile **3** along with the expected oxime diol **13** and triol **14**, although in different proportions (Scheme 4B). Nitrile formation was not detected in a control experiment (same substrates with *E. coli* JM109), thus suggesting oxime dehydratase activity of the TDO complex. However, because of the known *E/Z* isomerisation of benzaldoximes in protein preparations,[18] we cannot establish whether both isomers are substrates for TDO-mediated dehydration. The formation of **14** can be explained by reduction of the oxime moiety to the primary alcohol by an alcohol dehydrogenase in *E. coli*, according to previous reports.[18] In view of these results, we propose that pathway B2 of Scheme 3B describes the formation of benzonitrile.

The enzymatic transformation of oximes to nitriles has been described for Fe^{II}-containing enzymes such as aldoxime dehydratases,[19] and cytochrome P450 monooxygenases,[20] but to the best of our knowledge it has not been previously described for TDO or other Rieske dioxygenases. Further studies regarding this point will be reported in due course by our group.

Another possibility for the transformation of **8** into **3** is that hydroxyazide **9** could be transformed to benzonitrile by a Schmidt reaction[21] in the TDO active site, promoted by a proton transfer. However, the evidence of nitrile formation from benzaldoximes as well as the formation of **6** in the biotransformation of phenylethylazide makes this mechanism less probable and favours our proposed route. A possible explanation for the formation of **6** is coordination of the starting material with Fe^{II} to promote N_2 loss and thus formation of the corresponding metal–nitrene complex (Scheme 5). Rearrangement of the iron(IV)–nitrene complex would produce the corresponding imine, which is unstable in water and produces the aldehyde (Scheme 5B). As stated previously, reductases in *E. coli* could reduce this aldehyde to the phenethylic alcohol, which is a known substrate of TDO, thus finally giving triol **6**.[10e] The low overall yield for this transformation could be explained by the fact that the initial coordination to Fe would not be as strong as in complex **9**, as there is no OH group to produce a bidentate ligand.

In summary, we present the first example of enzyme-catalysed conversion of an alkyl azide into a nitrile. A proposed mechanism for this transformation by the TDO complex involves initial benzylic monooxygenation, followed by nitrene-mediated rearrangement to form an oxime, which is further dehydrated to afford the corresponding nitrile. This oxime-dehydration activity has not previously been reported for Rieske dioxygenases. The suggested iron-catalysed nitrene generation is being studied in our group as it could be of great importance and might contribute to the development of novel enzymatic C–H amination strategies of organic compounds,[22] a field that has been recently developed for P450 enzymes[17,23] but not yet for Rieske dioxygenases.

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Keywords: azides · biocatalysis · dioxygenases · oxidation · Rieske

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