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The Mechanism of Formation of N-Formylkynurenine by Heme Dioxygenases

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ABSTRACT: Heme dioxygenases catalyze the oxidation of L-tryptophan to N-formylkynurenine (NFK), the first and rate-limiting step in tryptophan catabolism. Although recent progress has been made on early stages in the mechanism, there is currently no experimental data on the mechanism of product (NFK) formation. In this work, we have used mass spectrometry to examine product formation in a number of dioxygenases. In addition to NFK formation (m/z = 237), the data identify a species (m/z = 221) that is consistent with insertion of a single atom of oxygen into the substrate during O2-driven turnover. The fragmentation pattern for this m/z = 221 species is consistent with a cyclic amino acetal structure; independent chemical synthesis of the 3a-hydroxyprolyloindole-2-carboxylic acid compound is in agreement with this assignment. Labeling experiments with 18O2 confirm the origin of the oxygen atom as arising from O2-dependent turnover. These data suggest that the dioxygenases use a ring-opening mechanism during NFK formation, rather than Criegee or dioxetane mechanisms as previously proposed.

INTRODUCTION

Tryptophan is essential for all mammalian systems, and tryptophan catabolism is regulated through the L-kynurenine pathway. The first, rate-limiting step of the kynurenine pathway is the oxidation of L-tryptophan to N-formylkynurenine (NFK, Scheme 1A). This is an O2-dependent process and is catalyzed by one of two heme enzymes: tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3 dioxygenase (IDO). The mechanism of tryptophan oxidation is of essential interest from a clinical perspective because tryptophan catabolism generates a number of secondary metabolites—such as 3-hydroxyanthranilic acid, anthranilic acid, 3-hydroxykynurenine, and quinolic acid—that are implicated in a wide range of neurological disorders, cataract formation, and suppression of T cell proliferation. In this sense IDO and TDO are emerging as important drug targets because they have the power to regulate the supply of tryptophan (Trp) and other kynurenine pathway metabolites. In consequence, the development of dioxygenase inhibitors is ongoing (with some compounds, such as 1-methyl-tryptophan (Me-TRp), in clinical trials).

Despite years of investigation going back as far as the 1930s, the reaction mechanism of the dioxygenases is still not clarified. Early studies concluded that the mechanism involved base-catalyzed deprotonation of the indole NH group as the initiating step (Scheme 1B), and were based on an assignment of Me-Trp as an inhibitor. It has emerged that there are numerous difficulties with this mechanism, however. Structural data are not consistent with the proposal since not all dioxygenases—human indoleamine 2, 3-dioxygenase being one example—contain an active site base (presumed to be a histidine). And, in cases where histidine is present, mutagenesis studies do not support an essential role for it. This led to an alternative proposal that did not require an obligate base (Scheme 1B). However, both proposals are problematic because neither is consistent with the chemistry of indoles (which do not react by loss of the indole proton). That deprotonation of the indole proton might be a red herring was first suggested from computational work but the experimental evidence has so far been from mass spectrometry which has shown that Me-Trp is not an inhibitor (as previously suggested) but a slow substrate. Since the indole proton is missing in Me-Trp (Scheme 1A), the logical conclusion is that the mechanism cannot involve its loss. Recent ENDOR data and computational work support this idea.

The later parts of the mechanism leading to formation of NFK are, as yet, unclarified. The literature describes either Criegee or dioxetane rearrangements (Scheme 1C), but there is no experimental evidence for either mechanism. Recent resonance Raman work has suggested that the mechanism may proceed by sequential insertion of oxygen. In the present paper, we have examined product formation in a number of different heme dioxygenases under turnover conditions. We find evidence, from mass spectrometry data, for insertion of a single oxygen atom into the substrate. This is not consistent with widely cited proposals for the concerted addition of both oxygen atoms.

Supporting Information

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An alternative mechanism is proposed for formation of N-formylkynurenine.

**MATERIALS AND METHODS**

Human TDO (hTDO), human IDO (hIDO) and X. campestris TDO (xTDO) were prepared as previously described. For LC–MS experiments, enzyme (1–5 μM), O2 and l-Trp (1 mM, Sigma) or 1-methyl-l-tryptophan (Me-Trp, 0.5 mM, Aldrich) were incubated in a sealed vessel for varying amounts of time (10–120 min), with conditions as previously described. The reducing system in all cases was ascorbate/methylene blue, as for normal dioxygenase assays, or in some cases with dithionite-reduced hTDO with O2 and l-Trp (1 mM) in 50 mM Tris-HCl buffer, pH 8.0 (25.0 °C). Steady-state formation of NFK was monitored at 321 nm using a Varian Cary 50 Probe UV–visible spectrophotometer with a 1 cm light path. Reactions were terminated by the addition of 30% (v/v) trichloroacetic acid although control reactions were also carried out in which TCA was not used. NFK was monitored at 321 nm using a Varian Cary 50 Probe UV–visible spectrophotometer with a 1 cm light path. For 18O2 experiments, all buffers and solutions were purged with nitrogen and then left to equilibrate in the glovebox for at least 2 h. Samples for the 18O2 experiments were prepared by bubbling 18O2 (98%, CK Gas Products Ltd.) into the assay mixture and the reaction initiated by the addition of enzyme. For reactions carried out in 18O-labeled water, all solutions were prepared using H218O (98%, CK Gas Products Ltd.) and the assays carried out as outlined above. Reactions were terminated by the addition of 30% (v/v) trichloroacetic acid as described above. LC–MS analyses used a Micromass Quatro LC spectrometer and samples were run with acetonitrile/H2O (0.1% formic acid) as the solvent (in a ratio of 10:90 or 20:80) on a C18 column. The 3a-hydroxypyrroloindole-2-carboxylic acid compound, referred to as the cyclic amino acetal in this paper (see Scheme 2, species Y), was synthesized (>95% purity, assessed by NMR) via dye-sensitized photooxygenation of L-tryptophan. NMR and mass spectrometry data are given in the Supporting Information (SI).

**RESULTS**

**Mass Spectrometry Analyses.** In our LC–MS analyses looking for NFK (m/z = 237) formation in a number of dioxygenases, a peak eluting with m/z = 221 has been routinely observed in addition to the expected peak at m/z = 237. A representative example is shown in Figure 1 for hTDO, but similar data have been obtained for other bacterial (xTDO) and mammalian (hIDO) enzymes under a range of conditions. Here, the products from the reaction of hTDO with O2 and l-Trp were examined using LC–MS (Figure 1A–D). Using selected ion monitoring (Figure 1A), the elution profile revealed a peak eluting with a mass at 221 (Figure 1B). This is consistent with insertion of a single oxygen atom into the substrate (M1 = 204; Table S1, SI). The observation of a peak at m/z = 221 did not depend on the reaction time (varied between 10 and 120 min), and is not a feature of the TCA precipitation treatment since the equivalent peaks at m/z = 221 (and m/z = 237) are also observed when TCA is not used. Nor is it a contaminant from the adventitious reaction of Trp with O2 under aerobic conditions, because no such peaks (either at m/z = 221 or m/z = 237) are observed for steady state assays obtained in the absence of enzyme. This is expected since Trp is only reactive with O2 in the presence of a sensitizer; in fact, this is used for the chemical synthesis of 3a-hydroxyproloindole-2-carboxylic acid below. Samples of l-Trp alone (i.e., l-Trp in buffer under aerobic conditions) similarly showed no peaks at m/z = 221 or m/z = 237 (and NMR analysis of l-Trp itself showed no peaks from impurities, data not shown).

We speculated that this peak may originate from initial conversion of the substrate to the corresponding 2,3-epoxide, which had been initially identified by computational studies (but considered energetically unfavorable in the gas phase) and also implicated from resonance Raman work. The proposed 2,3-epoxide is a known compound but is unstable and undergoes facile conversion to the more stable cyclic aminoacetal (Scheme 2). Mass spectrometry data for this derivative are published, including the expected fragmentation patterns in MS/MS experiments, which provides a useful control compound for the enzymatic turnover experiments. In LC–MS/MS analyses of product formation as above, a peak eluting at the same position is observed (Figure 1C) and with m/z = 221, and the fragmentation pattern, Figure 1D, matches that predicted for
the cyclic aminoacetal shown in Scheme 2 (peak at 175: loss of COOH; peak at 203: loss of H₂O₂; peak at 157: loss of H₂O and HCOOH).²¹ We thus assign the 221 peak detected in our mass spectrometry experiments as arising from initial formation of the epoxide intermediate, in which one atom of oxygen is inserted into the substrate during turnover, followed by conversion to the more stable amino acetal compound under the conditions of our experiments.²² In these experiments, we can also isolate an ion with m/z = 237 (Figure 1E), which is assigned as arising from NFK formation (Mᵣ = 236, Table S1, SI) by comparison with previous work.¹³

**Synthesis of the Cyclic Aminoacetal Intermediate.** To compare the mass spectrometry data above with the authentic 3a-hydroxypteridindole-2-carboxylic acid compound (Scheme 2), we synthesized the compound (>95% purity, assessed by NMR) via dye-sensitized photooxygenation of L-tryptophan.²⁰ The compound was isolated as a mixture of two diastereoisomers in a ratio of 2:1, and the structure confirmed by NMR analysis (see SI). Both isomers have a cis-5,5-ring junction with the major isomer having a cis-relationship between the hydroxyl and carboxylic acid groups (these assignments were confirmed by NOE experiments). LC–MS analysis of this synthesized sample indicated that it had the same retention time and the fragmentation pattern (see Supporting Information) as the 221 peak observed in the hTDO reactions.

In control experiments, we found that the purified cyclic aminoacetal (as a mixture of isomers) was not turned over to product by any of the dioxygenases examined in this work (no identifiable product peaks in mass spectrometry). This would be consistent with observations from mass spectrometry that the 221 species is a minor product (with most of the substrate turned over to NFK, as judged by comparing intensities of peaks eluting from LC–MS). Since the epoxide itself is not an isolatable compound, it was not possible to spike the reaction with epoxide in the same way.

**Labeling Experiments Using ¹⁸O₂.** To confirm that the 221 peak was generated from O₂-dependent turnover, the reactions with l-Trp were repeated under the same conditions but using ¹⁸O₂ to assess whether the oxygen atom in the 221 species originated from O₂ (or ¹⁸O₂). The results of these LC–MS/MS experiments are shown in Figure 2 for hTDO with l-Trp and for hIDO with Me-Trp, but similar data have been observed for xTDO with l-Trp. For hTDO, a peak is observed with m/z = 223 (Figure 2A), which corresponds to insertion of a single atom of ¹⁸O into l-Trp (Table S1, SI). In this spectrum there is also a peak at m/z = 177, which corresponds to the ¹⁸O-equivalent of the m/z = 175 fragmentation peak reported above (loss of COOH, as above). An ion with m/z = 241 was also detected, Figure 2B, which corresponds to the mass of the NFK product carrying two atoms of ¹⁸O (m/z = 241, Table S1 (SI)). In the corresponding reactions of hIDO with Me-Trp and ¹⁸O₂, we also observe peaks eluting with m/z = 237 and m/z = 255 (Figures 2C,D), which are as expected for the ¹⁸O-labeled monoxygenated intermediate species and ¹⁸O-labeled N-formylmethylkynurenine product (Mᵣ = 254, Table S1 (SI)).²³

For the reaction of hTDO and ¹⁸O₂, we also observed a product peak with m/z = 239 for the reaction with l-Trp

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**Figure 1.** Formation of NFK formed during O₂-dependent turnover by hTDO. LC–MS and LC–MS/MS analyses of the products obtained from the O₂-dependent reaction of hTDO with L-Trp in the steady state. (A) Elution profile for selected ion chromatogram with m/z = 221. The peak eluting at t = 8.58 min is kynurenine. l-Trp elutes at t = 13 min. (B) Corresponding positive ESI mass spectrum for the product eluted at 5.72 min, showing a peak at m/z = 221. (C) Elution profile for the same peak (at 5.60 min in this elution profile) and (D) corresponding MS/MS spectrum, showing the peak at 221 and a fragmentation pattern corresponding to the structure proposed in Scheme 2. (E) Positive ESI mass spectrum for the product eluted at 7.02 min, showing a peak at m/z = 237 corresponding to NFK formation. For all enzymes examined, the intensity of the 221 peak is invariably lower than that for the 237 peak, but we have been unable to extract quantitative time profiles from the mass spectrometric analyses.
Me-Trp and $^{18}$O$_2$ ((C) fragmentation pattern for ion with one atom of $^{16}$O and one atom of $^{18}$O from reaction of (A) hTDO with L-Trp and $^{18}$O$_2$ ($^{16}$O$^{18}$O-NFK) and (B) hIDO with Me-Trp and $^{18}$O$_2$ ((C) fragmentation pattern for ion with $m/z = 237$, (D) fragmentation pattern for ion with $m/z = 255$).

Figure 3. Evidence for mixed incorporation of $^{18}$O and $^{16}$O in NFK in reactions with $^{18}$O$_2$. LC–MS/MS analyses of NFK ($m/z = 239$) carrying one atom of $^{16}$O and one atom of $^{18}$O from reaction of (A) hTDO with l-Trp and $^{18}$O$_2$ ($^{16}$O$^{18}$O-NFK) and (B) hIDO with Me-Trp and $^{18}$O$_2$ ($^{16}$O$^{18}$O-Me-NFK).

(Figure 3A), and in parallel experiments a peak with $m/z = 253$ for the reaction with Me-Trp (Figure 3B). In both instances, this corresponds to the mass of NFK carrying one atom each of $^{16}$O and $^{18}$O (i.e., $^{16}$O$^{18}$O-NFK and $^{16}$O$^{18}$O-Me-NFK, instead of the expected product carrying two atoms of $^{18}$O$_2$). It is known from other work$^{24-26}$ that both carbonyl oxygen atoms in NFK are exchangeable with solvent. In recent analyses, Ronsein and co-workers$^{26}$ have observed exactly the same ‘mixed’ species ($^{16}$O$^{18}$O-NFK, $m/z = 239$) for NFK formed using singlet O$_2$ and have used the fragmentation patterns to show that the two carbonyl oxygen atoms in NFK have different rates of exchange with solvent, with the carbonyl oxygen group of NFK (blue in Figure 4) exchanging faster than the carbonyl oxygen of the amide group (red in Figure 4).$^{26}$ In our experiments for hTDO, xTDO, and hIDO with $^{18}$O$_2$ (Figures 4A–C), the $m/z = 239$ peak for the original mixed $^{16}$O$^{18}$O species fragments in all cases to give $m/z = 222$ (loss of the NH$_3$ group of the side chain), 204 (loss of NH$_3$ then H$_2$O), and $m/z = 192$ (loss of NH$_3$ then C$^{18}$O from the carbonyl of the amide group).$^{26}$ This is consistent with initial formation of $^{18}$O$^{18}$O-NFK, with subsequent exchange of the carbonyl oxygen to give $^{16}$O$^{18}$O-NFK ($m/z = 239$) followed by fragmentation leading to loss of $^{18}$O (from loss of C$^{18}$O, red) and leaving $^{16}$O in the product (blue).$^{26}$ To verify this, we carried out a control reaction with $^{18}$O$_2$ but with the protein exchanged into H$_2$O-containing buffer. In these experiments, $^{16}$O$^{18}$O-NFK ($m/z = 237$) formed initially, followed by exchange (as evidenced by a peak at $m/z = 239$, Figure 4D), but the fragmentation pattern is different (peaks at $m/z = 222$ as above, 202 (loss of NH$_3$ then H$_2$O$^{18}$O), and 194 (loss of NH$_3$ then C$^{16}$O), Figure 4D). This is again consistent with faster exchange of the carbonyl oxygen with $^{16}$O (from H$_2$O$^{18}$O water), because the fragmentation (Figure 4D) now leads to loss of C$^{18}$O (red) from the carbonyl of the amide group (not $^{18}$O, as above in Figure 4A–C). Together, these experiments provide assurance that the peaks for the mixed $^{16}$O$^{18}$O-NFK species are secondary products derived from subsequent exchange reactions with water (during handling).

## DISCUSSION

The activation of atmospheric oxygen is a fundamental requirement in biology that all aerobic organisms have to overcome. The difficulty is that oxygen is intrinsically unreactive, both thermodynamically and kinetically. Heme iron is often used to overcome these difficulties, and Nature uses heme enzymes for a large number of quite different, and sometimes difficult, biological oxidations. The mechanism of O$_2$ activation and NFK formation in the heme dioxygenases has yet to be clarified.

### Unraveling the Dioxygenase Mechanism

The mass spectrometry data presented in this paper reveal the presence of a stable amino acetal species, assigned as originating from initial formation of an unstable 2,3-epoxide, and consistent with insertion of a single atom of oxygen into the substrate during O$_2$-driven reactions. This is in contrast to the originally proposed mechanisms$^{16}$ for conversion of l-Trp to NFK, Scheme 1C, but would agree with more recent evidence$^{16}$ from resonance Raman work in which sequential insertion of oxygen into the substrate has been suggested. We envisage two possible mechanisms (Scheme 3, steps 1a and 1b$^{17}$), both of which fit with our data and with the supposed formation of ferryl heme.$^{16,28,29}$ One route is through electrophilic addition (steps 1b and...
2b\textsuperscript{12,13}, which is well-known in indole chemistry. However, in this mechanism the bound O\textsubscript{2} is denoted as an electrophile which could be problematic since O\textsubscript{2} bound to ferrous heme is often formulated as a ferric-superoxide species\textsuperscript{30} An alternative suggested route\textsuperscript{12,16,31} is through a radical-mediated reaction and formation of ferryl heme (Scheme 3, steps 1a and 2a). This would be attractive if the ferrous oxy species exists as ferric-superoxide\textsuperscript{30,32} (for which there is evidence\textsuperscript{16}) and would at the same time overcome the thermodynamic barrier to the required activation of O\textsubscript{2} (by one-electron reduction). Recent computational work favors the radical addition mechanism.\textsuperscript{33} It is perhaps also worth remembering that heterolytic cleavage typically requires considerable charge separation; hence, there is a requirement for electrostatic stabilization by residues in the distal pocket (Arg for example in the peroxidases), and the dioxygenase structures solved so far do not reveal large numbers of charged residues close to the heme iron (although there is an active-site Arg involved in substrate binding\textsuperscript{18}). Regardless of which mechanism is used (Scheme 3, steps 1a or 1b), neither requires abstraction of the indole proton and would thus be in agreement with most of the recent data\textsuperscript{9,12–16,33}.

**Formation of NFK.** The later stages of the mechanism leading to formation of NFK have been proposed\textsuperscript{1,34} as occurring through either Criegee or dioxetane pathways (Scheme 1C). A singularly misleading aspect of the literature is that these proposals are based on early speculation\textsuperscript{34} rather than on experimental fact (formation of a peroxyindole species, usually generated photolytically, is an implicit requirement\textsuperscript{11,35} and we presume this would be difficult to generate under enzymatic conditions). The conditions of our turnover experiments and mass spectrometry analyses allow us to identify a cyclic aminoacetal derivative, which we assign as a byproduct of the reaction that originates from ring-opening of the initially formed (and unstable) 2,3-epoxide (Scheme 2). It is well-known that epoxides can undergo facile C\textsubscript{2}/C\textsubscript{3} bond cleavage (due to the adjacent nitrogen lone pair\textsuperscript{36}), and for this reason we propose that the amino acetal species arises from ring-opening of the initial epoxide (Scheme 2).\textsuperscript{37} By analogy, this indicates to us that a heterolytic (two-electron) mechanism, again involving ring-opening of the initial epoxide, may be the initiating step in the dioxygenase enzymes (Scheme 3, step 3). This would allow for reactivity of both L-Trp and Me-Trp, both of which are substrates.\textsuperscript{13} Direct attack of the ferryl heme on the substrate can then be envisaged (step 4), after which

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**Scheme 3. Mechanistic Proposal for NFK Formation in the Heme Dioxygenases**

\textsuperscript{a} Epoxide formation is envisaged by two possible routes, an electrophilic mechanism (step 1a) or by radical addition (step 1b): as we highlight in the Discussion, both routes can lead to formation of an epoxide, but neither mechanism has been definitely proven. Ring-opening is indicated in step 3 (leading to the species labeled as X in Scheme 2), but could be synchronous with step 4. Cleavage of the C\textsubscript{2}/C\textsubscript{3} bond and formation of NFK is subsequently straightforward. For further details see text. R = CH\textsubscript{2}CH(NH\textsubscript{3}\textsuperscript{+})CO\textsubscript{2}$. The amine group of the side chain could potentially form a hydrogen bond to the $O^\text{-}$ of the singly oxygenated intermediate.
a heterocyclic (two-electron) mechanism for the required cleavage of the C2–C3 bond is feasible (step 5).39

Comparisons with Other Heme Enzymes. We have found that the amino acetal is not, under the conditions of our experiments, turned over by the enzyme to NFK, but we note that the very same species (labeled X and Y in Scheme 2) have been proposed40 as intermediates in the PrnB enzyme (species X and Y as also shown in Scheme 2). In PrnB this leads to formation of the tricyclic (amino acetal) intermediate, without insertion of oxygen, whereas in the dioxygenases C2–C3 bond cleavage and further O atom insertion is the preferred route.

The proposed lack of protons in the dioxygenase active site14 might be a controlling factor in discriminating one pathway against the other.

Epoxidation of substrates is, of course, well-known in the P450s41,42—in this case through direct reaction with the more powerfully oxidizing Compound I intermediate—but for indole compounds hydroxylation42 rather than epoxidation33,44 is more common. Drawing on this, we considered other mechanisms for NFK formation involving H atom abstraction from different positions on the substrate. Direct H atom abstraction from the C2–H group of the epoxide by Compound II was considered (Scheme S1A, SI) but subsequent conversion to NFK is not easily visualized. The most plausible alternative was considered to involve abstraction of the indole NH (which has precedent in P450 chemistry41,42) and is attractive because it leads to NFK formation through a simple and chemically convincing radical mechanism, Scheme S1B (SI). However, this mechanism has the disadvantage that it does not accommodate the known reactivity of Me-Trp,13 and we thus considered it less likely because Me-Trp and l-Trp are, at this stage, presumed to react through similar mechanisms.

There is logic in the idea that the shared heme structure used across all heme enzymes, together with the similarity of their reactions with O2 or its derivatives, means that there may be common patterns of reactivity across the entire family. Typically, other O2-dependent oxidative enzymes (e.g., P450s, NO synthase) react with O2 or derivatives of it (H2O2 in the peroxidases) to give an oxidized Compound I intermediate. Irreversible formation of Compound I is a reaction that the dioxygenases apparently need to avoid because formation of Compound I and recycling of the heme demands both protons (for evolution of water) and a continuous supply of electrons, neither of which is known to be required for dioxygenase turnover. Quite how such discrimination is achieved in the dioxygenases has yet to be established, but regulation of the reactivity of the ferrous-oxy species by limiting the supply of protons/electrons to the heme would be one solution to the problem. In this sense, the dioxygenases are unique because, rather than using the same oxidizing intermediate, they appear to use different heme intermediates for insertion of the two atoms of oxygen. This may be one of the key controlling features that helps the dioxygenases to specialize in NFK formation.

ASSOCIATED CONTENT

Supporting Information. Calculated masses for species observed in mass spectra (Table S1), alternative mechanisms (Scheme S1), NMR and MS data for the cyclic amino acetal compound, and a full citation for reference 18. This material is available free of charge via the Internet at http://pubs.acs.org.

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(22) An alternative explanation is that the 221 species is derived from a small substoichiometric amount of off-pathway epoxide intermediate (perhaps a feature of the way we conducted the mass spectrometry experiments) that forms during catalysis, whereas the majority of the active sites proceed through another, productive mechanism (producing just NFK). We cannot categorically rule out this possibility, although we have observed the 221 species under different experimental conditions for turnover and with different enzymes, both with and without TCA treatment.
(23) In these 18O2 experiments with ω-Trp, we also see minor peaks eluting with masses of m/z = 237 and m/z = 221, although the presence/absence of these peaks and their intensities vary from sample to sample. These peaks correspond to the 18O2 “equivalents” of the m/z = 241 and m/z = 223 peaks as described above, and most likely arise from trace amounts of 18O, introduced during handling and/or exchange processes described below.
(27) It is not known whether addition occurs at C2 or C3 of the substrate; both options are feasible for both mechanisms in Scheme 3 (but we only show one in each case).1,13,14,26