CYTOCHROME P-455 COMPLEX FORMATION IN THE METABOLISM OF PHENYLALKYLAMINES—IV.*

SPECTRAL EVIDENCES FOR METABOLIC CONVERSION OF METHAMPHETAMINE TO N-HYDROXYAMPHETAMINE.

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Abstract. Incubation of liver microsomes from phenobarbital-treated rats with N-methylamphetamine (1) and its N-oxidized congeners N-hydroxy-N-methylamphetamine (2), N-methylene-1-phenyl-2-propylamine N-oxide (3a) and N-(1-phenyl-2-propylidene)methylamine N-oxide (3b) gave rise to the formation of cytochrome P-450 product complexes characterized by maximum absorbances in the 453-457 nm region. Compounds 1, 2 and 3a showed maximum absorbances at 456 nm and for 2 and 3a both the rate and extent of complex formation was increased several fold over those of 1, with the complexing activity being about 90 per cent of that of N-hydroxyamphetamine (4a). Contrary to 1, 2, 3a and 4a. nitrone 3b showed its maximum absorbance at 453 nm and the spectral perturbations were identical to those seen with N-hydroxymethylamine (4b). Demethylation of 1 and 2, as monitored by formaldehyde production, showed good correlation with the complex formation. From the results it seems safe to conclude that 3a, formed after metabolic N-oxidation of N-methylamphetamine (1), undergoes further conversion to N-hydroxyamphetamine (4a), the latter being the ultimate precursor to the ligand forming the cytochrome P-455 complex. The results substantiate the notion that there is a preference for the formation of nitrones related to 3a rather than 3b during the metabolism of N-alkylamphetamines. Thus, in addition to α -carbon oxidation, N-oxidation is indicated as a route instrumental to the metabolic demethylation of N-methylamphetamine.

Since the discovery that a number of primary aliphatic amines, related to amphetamine, are able to form cytochrome P-450 product complexes during their metabolism [1], quite a bit of information has been gained as to the nature of these complexes. There is little doubt that N-oxidation is a prerequisite for complex formation [2-4] and several lines of circumstantial evidence indicate the nitroso congeners to be the ultimate ligands [4-6]. Complex formation also occurs during the NADPH-dependent metabolism of several secondary amines related to amphetamine [1, 7] but the mechanism of this reaction has not been investigated. In a previous publication [8] we indicated that N-oxidation seems to be a prerequisite for complex formation with the secondary amine norbenzphetamine (1, $R = C_0H_5$, Fig. 1). Moreover, in three recent publications [9-11] secondary hydroxylamines (2, Fig. 1) as well as nitrones (3a, Fig. 1) have been identified as in vitro metabolites of N-methylamphetamine (1, R = H in Fig. 1), N-ethylamphetamine $(1, R = CH_3)$ and norbenzphetamine (1, $R = C_6H_5$).

These findings warranted further investigation of the various N-oxidized products formed following the metabolism of secondary amines and as part of our studies on N-hydroxylation and cytochrome P- 455 complex formation [2, 4, 8], we investigated the occurrence of complex formation during metabolism of N-methylamphetamine and in this report describe the results of a study examining the complexing activity of various N-oxidized congeners (1–4b, R = H in Fig. 1).

MATERIALS AND METHODS

Instrumentation

Mass spectra (MS) were recorded on an LKB 9000 mass spectrometer using the direct probe technique. The ionizing potential was 70 eV, the trap current $60~\mu\text{A}$ and the accelerating voltage 3.5~kV. The temperature of the ionic source was 270° .

Ultraviolet (u.v.) and infrared (i.r.) spectra were recorded on a Beckman 25 and a Perkin-Elmer 157 G spectrophotometer, respectively. Nuclear magnetic resonance (NMR) spectra were run on a Perkin-Elmer R 12 spectrometer using C^2HCl_3 solutions. Chemical shifts are expressed in p.p.m. (δ) with tetramethylsilane as the internal marker. Microsomal complex formation was determined by difference spectroscopy using an Aminco DW-2 spectrophotometer.

Analytical thin layer chromatography (TLC) was done on precoated silica gel (60 PF, Merck) plates. Preparative TLC was performed with Merck's silica gel 60 PF, spread on glass plates to a thickness of 1 mm. Linear regression analysis was done on a Hewlett Packard HP 9810A calculator.

^{*} For part III see ref [8]. Presented in part at the Sixth European Work-Shop on Drug Metabolism, Leiden, June 1978.

Fig. 1. Possible oxidation routes for N-alkyl-1-phenyl-2-propylamines (N-methylamphetamine, 1, R = H).

Chemicals and synthesis

N-Methylamphetamine (1) was supplied by ACO Läkemedel and N-methylhydroxylamine-HCl (4b) was purchased from Fluka AG. The synthesis of N-hyroxyamphetamine (4a) has previously been described [12]. N-Hydroxy-N-methylamphetamine (2) was prepared according to Coutts et al. [9] and pure samples of nitrones 3a and 3b were obtained by modifications of their methods.

N-Methylene-1-phenyl-2-propylamine N-oxide (3a). To a solution of N-hydroxyamphetamine (4a. 500 mg, 3.3 mmol) in ethanol (99%, 10 ml) was added NaHCO3 (554 mg, 6.6 mmol). An excess of gaseous formaldehyde, generated by heating paraformaldehyde, was led into the reaction vessel and the mixture was left at 40° for 5 hr. The solvent was evaporated in vacuo and the residue treated with dry ether. Evaporation of the ether yielded a colorless oil which was repeatedly treated with a mixture of ether-ligroin. After evaporation of the solvent mixture, a final yield of 90 per cent of pure 3a was obtained. TLC developed in CHCl3:MeOH, 8:1 v/v showed only one spot with $R_t = 0.49$, indicating that no N-hydroxyamphetamine (4a, $R_t = 0.39$) was present. NMR, i.r. and MS-data were in good agreement with those reported by Coutts et al. [9]. u.v. λ_{max} at 230 nm. The oil was stored under nitrogen at -20° until used in the biological experiments.

N-(1-Phenyl-2-propylidene)methylamine N-oxide (3b). As described by Coutts et al. [9], a benzenewater mixture containing N-methylhydroxylamine—HCl (4b, 2.5 g, 30 mmol) and 1-phenyl-2-propanone (810 mg, 6 mmol) was refluxed in a Dean Stark apparatus until no more water was collected. Evaporation of the benzene in vacuo yielded a straw colored oil. On TLC (CHCl3:MeOH, 8:1 v/v), the oil gave two major ($R_f = 0.31$ and 0.25) and two less abundant spots, one of them ($R_f = 0.72$) corresponding to 1-phenyl-2-propanone. About 300 mg of the

crude oil was subjected to preparative TLC in a developing system of CHCl₈:MeOH (8:1 v/v). A broad zone corresponding to 3b was scraped off, extracted with 20% methanol in chloroform and the solvent was evaporated under dry N₂ to yield 80 mg of a colorless oil. MS:m/e (per cent relative abundance) 163 (M+, 100), 146 (58), 131 (96), 130 (19), 117 (32), 115 (26), 105 (37), 91 (51) and 56 (63). NMR: 7.33 (S,5H,Ar), 4.00 (S,1H,C $\underline{\text{H}}_2$), 3.78(broad S, 4H, CH₂ and N-CH₃), 2.16 and 1.98 (two S, 3H assignable to two non-equivalent CH₃). IR- ν_{max} (film). Strong to medium bands at: $\overline{1150}$ and 1180 cm⁻¹, either of which can be assignable to the N=O-stretching, 1580 cm⁻¹ (C = N). UV: λ_{max} at 228-230 nm. The oil was stored under nitrogen at -20° until used in the biological experiments.

All chemicals used in the enzymic studies were of analytical grade. NADPH and bovine serum albumin were purchased from Sigma Chemicals. The water used was doubly distilled.

Stability studies of nitrones 3a and 3b

To ascertain pure substrates for the enzymic studies, the hydrolytic properties of nitrones 3a and 3b were investigated. Hydrolyses were performed by stirring freshly prepared $10^{-4}-10^{-2}$ M solutions of the nitrones in open vessels at 25°. The medium was 20% ethanol and the appropriate buffer to a final ionic strength of 0.1. The hydrolysis was monitored by means of u.v. with spectra being repeatedly recorded in the 210–300 nm range.

Enzymic studies

Hepatic microsomes were prepared from phenobarbital treated (three daily i.p. injections of 80 mg/kg and 40 mg/kg on the fourth day) male Sprague–Dawley rats weighing 220–240 g, and were isolated by gel filtration [13] to ensure complete removal of hemoglobin [14].

Spectrophotometric measurements were done at 25°. The microsomal concentration was 1 mg protein/ml in 50 mM potassium phosphate buffer pH 7.5. Microsomal suspension (5.8 ml) containing substrate as indicated and 10 mM MgCl2 was divided between the cuvettes and the base line in the range 390–500 nm was recorded. Water (0.1 ml) was added to the reference cuvette and the enzymic reaction was started by addition of 1 mg NADPH in 0.1 ml water to the sample cuvette. The change of absorbance $\Delta A_{(455-490)} \cdot \mu \text{mol P-}450^{-1}$ as a function of time was determined by repetitive scanning. $K_{m(app)}$ and $V_{\text{max(obs)}}$ were obtained by linear regression analysis of the Lineweaver-Burke plots with $V_{\text{(obs)}}$ being equal to the initial rate. The correlation coefficients were ≥ 0.98 for the substrate ranges studied.

In one set of experiments demethylation of 1 and 2 was monitored by formaldehyde production measured by the method of Nash [15]. The incubation mixture was identical to that used for the spectral studies. The incubation was terminated by the addition of 1.5 ml of 5.1% perchloric acid. Controls were obtained by incubating 1 and 2 in the presence of co-factors and inactivated microsomes (heated at 90° for 5 min).

Protein concentration [16] and cytochrome P-450 [17] were determined according to standard procedures.

RESULTS AND DISCUSSION

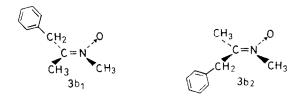
Nitrones 3a and 3b—Chemical considerations

Coutts et al. [9] have fully characterized nitrone 3a but not nitrone 3b. They prepared and used 3b as a mixture of products, containing about 70 per cent of the desired nitrone, without further purification. Our studies used the nitrones as substrates for enzymic studies and required pure substrates. While nitrone 3a could be obtained directly sufficiently pure, nitrone 3b had to be further purified. This was achieved by preparative TLC.

The purified oil showed an NMR-spectrum with two non-equivalent CH₃-groups exhibiting signals of equal intensity indicating a mixture of two isomers. Evidently the purified fraction constituted an equimolar mixture of the E- and Z-forms of the nitrone [18] (Fig. 2), with stereochemical properties analogous to those found for 1-phenyl-2-propanone oxime, an amphetamine metabolite (5b, Fig. 2) [19].

The oxime (5b) exhibits an NMR-spectrum very similar to that of 3b [12]. Purified 3b showed a mass spectrum in qualitative agreement with that of the impure product [9] but the relative abundances of certain fragments were quite different. The product contained no detectable amounts of the 3a isomer.

Both nitrones are stable for weeks when stored under N_2 at -20° . They are also surprisingly stable towards chemical hydrolysis at pH 7.5. Thus, preliminary kinetic studies indicate that in phosphate



$$\begin{array}{c|c} CH_2 & CH_3 \\ C=N & C=N \\ CH_3 & C=N \\ CH_2 & OH \\ 5b_1 & 5b_2 \end{array}$$

Fig. 2. Structures of the (E)- and (Z)-isomers, 3b₂ and 3b₁ respectively, of N-(1-phenyl-2-propylidene)methylamine N-oxide and their relationship to the (E)- and (Z)-isomers, 5b₁ and 5b₂ respectively, of 1-phenyl-2-propanone oxime (phenylacetoneoxime).

buffer at pH 7.5 and 25°, 3a and 3b have half-lives of at least 22 hr. Both nitrones are, however, very sensitive towards alkaline as well as acidic hydrolysis. Further details on the chemical stability of these and related nitrones [8, 20] will be published elsewhere.

Cytochrome P-450 product complex formation

Typical difference spectra obtained during NADPH-dependent metabolism of *N*-hydroxy-*N*-methylamphetamine (2) and the nitrone 3a are shown in Fig. 3. The spectra are indistinguishable and they are in turn identical with that obtained during NADPH-dependent metabolism of the primary hydroxylamine, *N*-hydroxyamphetamine (4a) [21].

Among the various substrates investigated in this study (1-4b) differences are seen both in the rate and extent of complex formation (Fig. 4 and Table 1). N-Methylamphetamine (1) is a relatively poor substrate with a significant lag phase prevailing during the first minute of incubation, and in this respect 1 behaves like amphetamine [1, 2]. For N-hydroxy-N-methylamphetamine (2) and nitrone 3a both the rate and extent of complex formation are increased several fold over those of 1, while between themselves 2 and 3a show only small differences. The extent of complex formation (Table 1) for 2 and 3a is also approaching that obtained with N-hydroxyamphetamine (4a), the most active substrate of those investigated. N-Hydroxy-N-methylamine (4b) and nitrone 3b are both considerably less active than their analogues 4a and 3a. It should also be noted that precise spectral measurements revealed a blue shift in the maximum absorbance of 3-4 nm for 4b and 3b with respect to all the other compounds (Fig. 5).* No complex formation occurred with any of the substrates when sodium dithionite was substituted for NADPH [3, 4], but once formed all the complexes investigated were stable to the presence of dithionite.

^{*} In a previous report Mansuy *et al.* [6] indicated a λ_{max} of 455 nm for the complex with *N*-hydroxymethylamine (4b). The difference between 4a and 4b noted by us has, however, been confirmed by an independent reinvestigation performed by Dr. Mansuy (private communication).

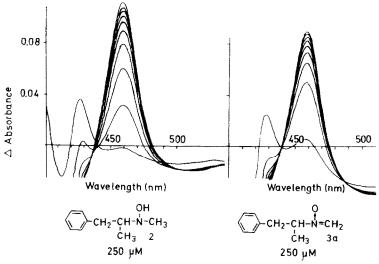


Fig. 3. Difference spectra produced during NADPH-dependent microsomal metabolism of N-hydroxy-N-methylamphetamine (2) and N-methylene-1-phenyl-2-propylamine N-oxide (3a). The time difference between each scan is ~ 40 sec. For the experimental conditions see Materials and Methods.

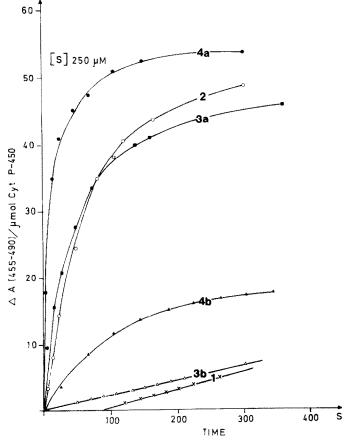


Fig. 4. Cytochrome P-450 complex formation by compounds 1–4b in microsomes isolated from phenobarbital treated rats. Microsomes (2 μ M of cytochrome P-450) were incubated in the presence of *N*-methylamphetamine (1), *N*-hydroxy-*N*-methylamphetamine (2), *N*-methylene-1-phenyl-2-propylamine *N*-oxide (3a), *N*-(1-phenyl-2-propylidene)methylamine *N*-oxide (3b), *N*-hydroxyamphetamine (4a) and *N*-hydroxymethylamine (4b) respectively under the conditions described in Materials and Methods. All substrate concentrations were 250 μ M. One representative experiment out of 4. For standard deviations cf. Table 1.

Compd.	${V_{ m max(obs)}}^*$	$K_{m(\mathrm{app})}\mu M$	Range μΜ	Amount of complex		
				[s] μM	$\Delta A_{ m max}$ †	Time (sec)
1				50	8.3 (1)	980 (1)
1			**********	250	$18.6 \pm 1.0(3)$ ‡	$937 \pm 18(3)$ ‡
1			-	5000	$22.7 \pm 2.3(2)$	$1036 \pm 17(2)$
1			45,040	10,000	20.9 (1)	1047 (1)
2	$33.4 \pm 1.3(3)$ ‡	$19 \pm 4(3) \ddagger$	2.5-100	250	54.4 ± 2.9 (2)	$835 \pm 30(2)$
3a	$55 \pm 13 \ (3) \ddagger$	$59 \pm 6 (3) \ddagger$	5.0-100	250	51.8 ± 1.3 (2)	$868 \pm 72 (2)$
4a§	$187 \pm 14 \ (3)$ ‡	$11.7 \pm 0.6 (3) \ddagger$	1.0-100	250	$59.4 \pm 1.7(2)$	$543 \pm 57 (2)$

Table 1. The rate and extent of complex formation in NADPH-dependent metabolism of 1, 2, 3a and 4a

For the experimental particulars see Materials and Methods.

In agreement with what has been reported for the primary phenylalkylamines [4, 6], N-oxidation seems a necessity for complex formation with the secondary amines. This was also indicated in a recent study with norbenzphetamine [8]. Thus N-methylamphetamine (1) is not able to form a complex without the prerequisite of metabolism, and the enhanced rate and extent of complex formation seen with 2 suggest this compound to be closer to the complex forming metabolite. In the case of primary amines, the nitroxides or nitroso species are regared as the ultimate ligands [2-6]. Further oxidation of 2 (Fig. 1) could result in the formation of either or both of the nitrones 3a and 3b, both being counterparts to the nitroso compounds. One could then speculate whether either of these is the actual ligand, but contrary to the nitroso compounds, nitrones 3a and

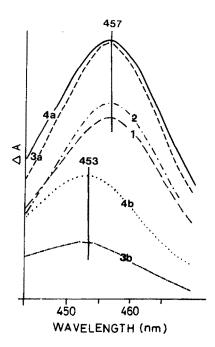


Fig. 5. Difference spectra produced during NADPHdependent microsomal metabolism showing the blue shift in the Soret peak position for 3b and 4b with respect to 1, 2, 3a and 4a.

3b are unable to form complexes without the prerequisite of metabolism. The possibility of a secondary nitroxide (Fig. 6), formed after reduction of 3a or 3b, to act as the ligand, can also be excluded, because in the presence of dithionite both nitrones 3a and 3b should generate the very same nitroxide, i.e. 3a and 3b would be expected to exhibit identical spectral activities which is not the case (Fig. 5).

Reactions, other than reduction, of the nitrones (3a and 3b) at or close to their nitrogen center will involve their hydrolysis [22, 23] (Fig. 1), implicating the formation of the primary hydroxylamines 4a and 4b. This mechanism generates the very same intermediates as the metabolism of the primary amines and, as indicated above, the corresponding nitroso species will then be the ultimate ligands. Moreover as 2, 3a and 4a effect identical spectral perturbations, different from those caused by 3b and 4b (Fig. 5 and Table 1), the former and not the latter are the intermediates that relay the spectral properties seen with 2. In this context it could be noted that both isomers of the oxime 5b (Fig. 2) are devoid of complexing activities [3].

The implication of the mechanism outlined above is further indicated by the demethylation studies, which give direct evidence, coupling oxidation of 2 to complex formation. Thus demethylation of Nhydroxy-N-methylamphetamine (2), as monitored

Fig. 6. N-Methyl-1-phenyl-2-propylamine nitroxide as a common intermediate in the reduction of N-methylene-1phenyl-2-propylamine N-oxide (3a) and N-(1-phenyl-2-propylidene)methylamine N-oxide (3b).

^{*} ΔA (455–490) · cm⁻¹ · P-450 mM⁻¹ · min⁻¹. † ΔA (455–490) · cm⁻¹ · P-450 mM⁻¹.

 $[\]ddagger \pm S.E.M.$ (n).

[§] Cf [14, 21] $K_s = 33 \mu M$, $\Delta A_{\text{max}} = 60 (455-490) \cdot \text{cm}^{-1} \cdot \text{P-450 mM}^{-1}$.

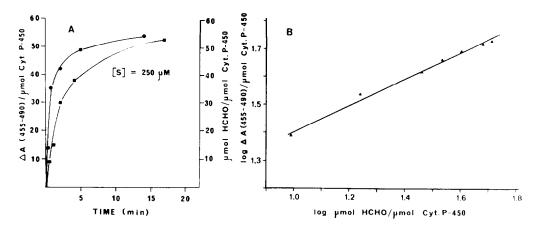


Fig. 7. Curves showing the correlation in rate between cytochrome P-455 complex formation () and formaldehyde production () during NADPH-dependent metabolism of N-hydroxy-N-methylamphetamine (2). One representative experiment out of 3. A: Rate is time curves. ε for the complex generated from N-hydroxyamphetamine (4a) is 65 mM · cm · [21]. Rather high blanks were obtained with 2 probably due to autoxidation of 2 during the assay conditions, cf. [23]. The blanks were compensated for by carefully performed control experiments. One series of controls were run for each time point. Inactivated microsomes (heated at 90° for 5 min) were used. The standard deviation between 9 controls was less than 11 per cent. For the experimental particulars see Materials and Methods. B: The log vs log relationship. Linear regression analysis gave a correlation of 0.99 ± 0.02.

All substrate concentrations were 250 μM.

by formaldehyde production, showed good correlation with the complex production (Fig. 7). Demethylation experiments could not be performed with 3a as the compound hydrolyzed during the assay conditions.

Although the results suggest a conversion of Nhydroxy-N-methylamphetamine (2) to N-hydroxyamphetamine (4a), with the nitrone 3a as an intermediate, they do not per se prove a metabolic conversion of N-methylamphetamine (1) to 4a by route of initial N-oxidation of 1. An alternative mechanism for metabolic activation of 1, involving conventional α -carbon hydroxylation and cleavage of the C-Nbond to produce amphetamine which in turn could undergo N-oxidation, should also be considered. The results from the demethylation studies performed with 1 repudiate the involvement of such a mechanism. Only small, but distinct, amounts of formaldehyde, up to about $10 \mu M$ were produced in the presence of NADPH. If the demethylated product constituted amphetamine, then no complex formation would be noticed, as 10 μ M of amphetamine is too low a concentration to generate detectable amounts of complex [4]. However, the amount of complex produced during metabolism of 1 at saturating substrate concentrations (Table 1) corresponds nicely to the amount produced $\Delta A_{\text{max}} 30-35$. by 10 µM of N-hydroxyamphetamine (4a). Accordingly, N-methylamphetamine (1) is most likely converted to 4a by route of initial N-oxidation followed by dealkylation. Moreover, in the recent work by Coutts et al. [9], 2 and 3a were established as two major in vitro metabolites of 1, 4a was present as a minor metabolite while, interestingly, 3b could not be detected at all. Similar results have been obtained also with norbenzphetamine [11], N-ethyl-and Npropylamphetamine [10, 24].

It should be stressed that only minute amounts of

the ligand are required to occupy the enzyme. Assuming a 1:1 ratio of ligand to cytochrome P-450. at a substrate concentration of 250 μM and an enzyme concentration of 2 μ M, less than a 1 per cent conversion of the substrate is required for 100 per cent complex formation. From the ΔA_{max} values in Table 1 and the absorption coefficient for the Nhydroxyamphetamine complex, $\varepsilon = 65 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ as calculated by Franklin [21], the amount of enzyme complexed in the presence of the various substrates ([S] = 250 μ M) can be estimated to 91 per cent (4a). 80 per cent (3a), 84 per cent (2) and 29 per cent (1). This shows that, at this substrate concentration. essentially all of the enzyme is complexed after metabolism of 2, 3a and 4a, while the metabolism of 1 ties up only about 30 per cent. This, in turn, gives an indication on the amount of N-methylamphetamine (1) converted to ligand forming metabolites [30 per cent of 2 μ M (enzyme concentration) = $0.6 \,\mu\text{M}$, which equals about 0.2 per cent of the substrate concentration. Increasing the substrate concentration did not significantly increase this number (Table 1).

In the conversion of N-methylamphetamine (1) to the ligand forming species, oxidation of 1 is the rate limiting step as indicated by the kinetic data (Fig. 4 and Table 1). The kinetics seen with 1 are, however, different from those found with 2, 3a and 4a, and are characterized by straight lines (e.g. in Fig. 4) at all substrate concentrations investigated. This finding indicates a feature of zero order kinetics which is subject to further investigation.

CONCLUSIONS

From the results of the present study it seems safe to conclude that the nitrone 3a, formed after meta-

bolic N-oxidation of N-methylamphetamine (1), undergoes further conversion to N-hydroxyamphetamine (4a). The latter is the ultimate precursor to the ligand forming the cytochrome P-455 complex. Our results also substantiate the notion [9–11], that there is a preference for the formation of nitrones related to 3a rather than 3b during the metabolism of amphetamines. In addition to α -carbon oxidation, N-oxidation is indicated as a metabolic route, in this case instrumental to metabolic N-denethylation, but more generally instrumental to N-dealkylations. Thus, the results reanimate the debate on the significance of N-oxidation in dealkylation reactions [25, 26].

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