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A dynamic combinatorial screen for novel imine reductase activity

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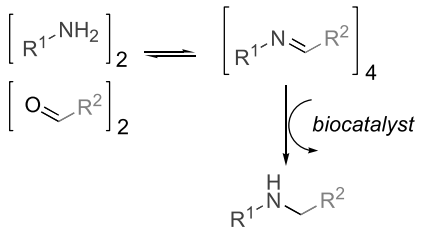
Abstract—New imine reductase activity has been discovered in the anaerobic bacterium *Acetobacterium woodii* by screening a dynamic combinatorial library of virtual imine substrates, using a biphasic water–tetradecane solvent system. Benzylidine aniline and butylidine aniline were reduced to the corresponding amines by caffeate-induced cells, whereas uninduced cells reduced butylidine aniline only. The reductions were detected despite side reactions that consumed some of the starting materials. The new screen can now be extended to discover synthetically useful imine reductases and enzymes that catalyse reactions for which biocatalytic equivalents of the chemical reactions have not yet been discovered.

1. Introduction

Combinatorial chemistry is a well-established tool for drug discovery. In particular, dynamic combinatorial approaches provide a simplified, rapid and versatile approach to discover new molecules that interact selectively with biological targets.[1] In drug discovery, a virtual library of candidate molecules is constructed by reversible self-assembly from basic component molecules. The active library constituents (e.g., selective enzyme inhibitors) are 'expressed' by binding to the biological target (e.g., the enzyme). The active constituents are 'locked in' so that they can be identified. Since the inhibitor is not modified by the enzyme, this is achieved by a chemical transformation or a change in reaction conditions. This quenches the reversible process and converts the active constituent to a stable chemical product[1,2] or stabilizes it sufficiently for it to be retrieved and characterized.[1]

Here, we report the first application of a dynamic combinatorial library to screen whole cell biocatalysts for a novel class of biotransformation, the reduction of imines to secondary amines. Virtual libraries of imines can be self-assembled from a mixture of component amines and aldehydes, and this has already been used to screen for enzyme inhibitors.[2,3] The key conceptual—and practically significant—advance is to use the enzyme substrate selectivity to perturb the equilibrium for self-assembly of the active library constituent and then to use the biocatalytic reaction to lock in the expressed constituent, so that the preferred imine is reduced to the corresponding secondary amine (Scheme 1).



Scheme 1.

A dynamic combinatorial screen for biocatalytic imine reduction would be extremely useful. Asymmetric reduction of ketimines, or related C=N containing compounds (hydroxylamines, hydrazones and oxime ethers), is an important target since this approach should provide a versatile strategy to synthesize α-chiral amines.[4]

Although chemical imine reductions are improving all the time,[5,6] biocatalysis may provide a route to enantiopure amines that are not yet accessible by chemical catalysis. However, imine reduction is one of a number of reaction classes for which there are no known enzymes that catalyse synthetically useful reactions,[7] but where there are no theoretical reasons why such enzymes should not exist.

Many laboratories have attempted to isolate useful imine reductases but with no success. A major problem is that imines tend to hydrolyse very readily in aqueous reaction systems, making it difficult to screen whole cell biocatalysts for imine hydrogenation. Furthermore, substrate instability means that living organisms are not exposed to imines in

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natural ecosystems, except as transient intermediates of metabolic pathways.[7a–d] Therefore, we cannot expect specialized enzymes to have evolved for imine metabolism. On the other hand, there may be enzymes that catalyse imine reduction fortuitously. This still presents problems, because we cannot predict their natural biological function or their substrate preference. This makes it extremely difficult to design suitable screens for imine reductases.

Dynamic combinatorial screens would be extremely effective in solving these problems. Thus, the target enzymes can 'choose' their preferred substrates from the virtual library and a very wide range of substrates can be screened simultaneously. This is ideal for detection of biocatalysts with unknown substrate preferences. Furthermore, the screen actually exploits the hydrolytic equilibrium, since the substrate selectivity of the enzyme forces the equilibrium towards generation of the preferred substrate by continuously converting it to the reduced product. Therefore, substrate instability is no longer a problem.

We developed the dynamic combinatorial screen using the strictly anaerobic bacterium, *Acetobacterium woodii*. We chose to use an anaerobe for screening for several reasons. Aerobic microorganisms and facultative anaerobes are more likely to hydrolyse the C=N bond than reduce it, because this produces aldehydes and amines which can be oxidized very easily.[14] In contrast, hydrogenation produces secondary amines, which are much more resistant to oxidative enzymatic attack. Secondary amines are either metabolized by oxidation to the imine[8] (which would be a futile cycle for imine oxidation) or by reductive cleavage to a primary amine, which only occurs in obligate anaerobes,[9] and is, therefore irrelevant to aerobic metabolism. Therefore, hydrogenation of imines is unlikely to occur in oxidative metabolic processes, and the search for imine reductases should be directed towards the reductive sector of metabolism.

Hydrogenation of organics is very favourable when the reaction serves as the electron-accepting process for growth of anaerobic bacteria.[10] This is a type of anaerobic respiration, where hydrogenation of the organic is analogous to the reduction of oxygen to water in aerobic respiration. The reactions are single step, occur at high rates and the hydrogenated products accumulate without further metabolism. Most importantly, the enzymes often have broad substrate ranges.[10] Therefore, if imine hydrogenation is to occur at all, it is most likely to occur as a fortuitous reduction catalysed by an enzyme involved in anaerobic respiration.

We used *A. woodii* in this study because this organism can reduce C=C double bonds as a respiratory process.[11] Our reason for this choice is that there is good chemical analogy between reduction of alkenes (of conjugated systems) and imines and we hoped that a C=C reductase might accept imines as fortuitous substrates. We wish to report the successful use of a combinatorial dynamic screen to identify a new imine reductase activity in whole cells of *A. woodii*.

2. Results and discussion

This work targeted the reduction of aldimines as a model for establishing the principle of this screening approach and the identification of new biocatalytic C=N reductions. The aldmines to be screened were chosen to include combination of alkyl–alkyl, alkyl–aryl and aryl–aryl components (from the carbonyl and amine components, respectively). Although, these would not yield chiral products, the use of aldehydes provides a very robust test of dynamic combinatorial screening for a number of reasons.

1. Substrate and product toxicity is a frequent complication in whole cell biotransformations and may hinder detection of target reactions in a screen. Aldehydes are more toxic than the corresponding ketones that would be needed for self-assembly of prochiral ketimines, since aldehydes react very readily with cellular amines (e.g., proteins, etc.). Therefore, use of the aldehydes provides a good test of the robustness of the approach.

2. Whole cells contain a multiplicity of enzymes, which might act on the starting materials or the components of the virtual library, and thus reduce the availability of potential substrates and cause the formation of side products. Aldehydes are more susceptible to biocatalytic side reactions than ketones, and thus provide a better indication of any problems that might be caused by competition between the target biotransformation and side reactions.

We produced *A. woodii* cells for use as the biocatalyst using growth conditions and methods for cell harvesting developed previously.[12] We used hydrogen as the electron donor for hydrogenation of the virtual library, since H_2 can be oxidized efficiently by the cells to yield the reduced cofactors needed for the biotransformation.[12b] The biocatalyst was grown in the presence and absence of the inducer, caffeate, to produce cell preparations containing and lacking the enzyme needed for C=C reduction,[12b,c] respectively. This would allow us to test whether or not the C=C reductase is responsible for any C=N reductions detected.

First of all, we confirmed that the biocatalyst had behaved as expected. Thus, the growth rate was 12% faster and the final

Table 1. Growth[a] and caffeate reduction[b] by *A. woodii* in the presence and absence of caffeate

Growth conditions	Growth rate (h^{-1})	Final biomass concentration ($g\,l^{-1}$)	Caffeate reduction rate (mmol h^{-1} kg dry $weight^{-1}$)
No additions	0.157	2.3	0
Caffeate	0.176	4.18	1615

[a] *A. woodii* was grown with or without caffeate (1 mM) and the growth rate and final biomass concentration were determined. The cells were then harvested from each culture and tested for reduction of caffeate (2.5 mM).
[b] Using harvested cells.

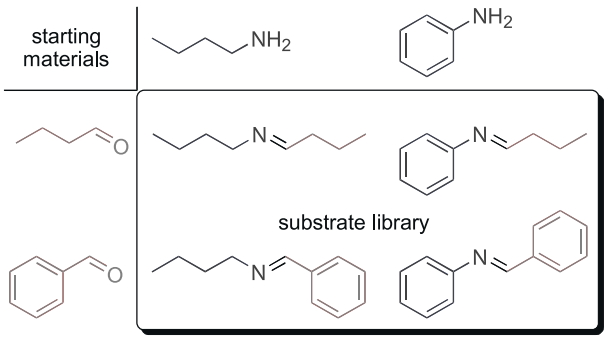

Figure 1. Virtual library of aldimines tested as substrates for *A. woodii*.

biomass concentration was 75% higher in the cultures grown with caffeate than in cultures grown in the absence of caffeate (Table 1). Similarly, the harvested cells could only reduce caffeate after growth in the presence of caffeate, confirming that reduction of C═C double bonds was inducible.[12b,c] It should be noted that we tested for caffeate reduction in the presence of tetradecane (phase ratio 0.2), since we planned to use a 2-liquid phase reaction system to deliver the virtual library, and we wanted to confirm that the cells were not affected by the solvent. In fact, caffeate reduction occurred at a slightly higher rate than reported previously with H_2 as electron donor in the absence of tetradecane,[12b] possibly because H_2 is more soluble in tetradecane than water.

The same preparations of harvested cells (induced and uninduced) were then tested for reduction of a virtual library of aldimines with the constituents shown in Figure 1. The constituents were derived from a mixture of aniline, benzaldehyde, butanal and butylamine. The two-liquid phase reaction system was used to ensure that all starting materials, virtual library constituents and any reaction products were in solution phase, and, thus, in contact with the biocatalyst.

Two reduced products, *N*-butylaniline and phenylbenzylamine, were formed in small quantities in reaction mixtures containing cells which had been grown with caffeate. Cells grown without caffeate produced only *N*-butylaniline (Table 2), but the concentration of *N*-butylaniline was much higher than with the cells grown in the presence of caffeate. Neither product was formed in reaction mixtures containing boiled cells or without any cells. This shows that reduction of the virtual library constituents was biocatalytic rather than chemical.

Only one constituent of the virtual library, benzylidine aniline, could be detected in any reaction mixtures. Thus, relatively large quantities of benzylidine aniline were present in control reaction mixtures containing boiled cells or without cells (Table 2). When active biocatalyst grown without caffeate was present, the concentration of benzylidine aniline was much lower, whilst this library component could not be detected at all in reaction mixtures containing cells that had been grown in the presence of caffeate. This trend reflects the formation of phenylbenzylamine, and further indicates that the cells were converting benzylidine aniline from the virtual library to the product.

Although *N*-butylaniline was produced by the biocatalyst, the starting material, butylidine aniline, was not detected in any reaction mixture. Butylidine aniline is less stable in water than benzylidine aniline,[13] and this may explain our inability to detect this library constituent. This confirms that the 'actual' substrate does not need to be present at detectable levels in the reaction mixture and that the presence of the 'virtual' substrate is sufficient to detect the biocatalytic activity.

Of the starting materials, only aniline could be detected in all of the reaction mixtures (results not shown). Benzaldehyde was detected in reaction mixtures containing boiled cells or no cells, but not with active cells. This suggested that the cells had consumed this substrate completely. Butanal and butylamine were not detected in any reaction mixtures, presumably because they could not be resolved from the tetradecane used as the solvent in the two-liquid phase system.

Table 2. Reduction of a virtual library by *A. woodii*[a]

Conditions for growth of biocatalyst	Biocatalyst preparation and no. of replicates	Library components, peak area×10^{-6}		Products, peak area×10^{-6}				Side products, peak area×10^{-6}			
		Benzylidine aniline		*N*-Butylaniline		Phenylbenzyl amine		Benzyl alcohol		Benzoic acid	
		C14	H_2O	C14	H_2O	C14	H_2O	C14	H_2O	C14	H_2O
With caffeate	Active (3)	0	0	0	0.065	0	0.0113	17.6	308	0	208
	Boiled (1)	835	0	0	0	0	0	1.7	86	0	44
	None (1)	729	0	0	0	0	0	0	31.3	0	13
No additions	Active (3)	6.03	8.13	0.267	0.307	0	0	0	391	0	71
	Boiled (1)	387	0	0	0	0	0	0	0	0	153
	None (1)	280	0	0	0	0	0	0	0	0	71

[a] *A. woodii* was grown with or without caffeate (1 mM), harvested and tested for reduction of a virtual library in a 2-liquid phase reaction system containing tetradecane and water (active). These were the same cell preparations as those used for the experiments shown in Table 1. Control reaction mixtures containing boiled cells (boiled) or no cells (none) were tested under identical conditions. The data are averages for the number of replicate reaction mixtures for each biocatalyst preparation shown in parentheses. The tetradecane (C14) and aqueous (H_2O) phases were analysed for library components, reaction products and side products. Data for each component detected are given for each phase as peak areas×10^{-6}.

Side products were also formed. Thus, benzaldehyde was converted to benzyl alcohol and benzoic acid in approximately equal quantities by biocatalyst that had been grown with caffeate (Table 2). In contrast, cells grown without caffeate produced mainly benzyl alcohol. Small quantities of benzyl alcohol and benzoic acid were also formed in control reaction mixtures containing boiled caffeate-grown cells, but only benzoic acid was formed in the presence of boiled cells grown without caffeate. This suggests that the side reactions were partly non-enzymatic and that some benzyl alcohol formation was caused by chemicals associated with caffeate-grown cells. In control reaction mixtures without cells, one reaction mixture contained smaller quantities of both benzyl alcohol and benzoic acid, whilst one contained only benzoic acid, again confirming that the reactions were partly non-enzymatic.

3. Conclusions

Reduction of a virtual library of aldimines was achieved using whole cells of *A. woodii*. Two constituents of the library (benzylidine aniline and butylidine aniline) were expressed in the presence of cells that were induced for caffeate reduction. In contrast, only butylidine aniline was expressed in the presence of uninduced cells. This suggests that *A. woodii* may contain two imine reductase activities, one of which (the benzylidine aniline reductase) is coinduced or identical with the caffeate reductase, and one (the butylidine aniline reductase) that is produced constitutively. The quantities of product were very low in each case, so it is possible that screening a larger virtual library would reveal better substrates. Once these are identified, further work will be needed to characterize these novel imine reductases.

In this study, product formation was detected using a whole cell biocatalyst. This demonstrates that the physical barrier of the bacterial cell membrane does not hinder the generation and biotransformation of a virtual library. Even though toxic substrates were used and the cells catalysed side reactions, low concentrations of product could still be detected. The most remarkable aspect is that the induced cells produced phenylbenzylamine even though one of the precursors, benzaldehyde, was also converted to benzyl alcohol and benzoic acid. This demonstrates that biocatalytic lock in of the reaction products perturbs the equilibrium sufficiently to direct the flow of precursors to the target biotransformation even in the presence of side reactions.

In this preliminary study, we have demonstrated that it is feasible to use dynamic combinatorial screens to identify new biocatalytic activities in whole cells, even with toxic substrates and in the presence of side reactions. It will now become possible to screen a wider range of potential biocatalysts with larger virtual libraries of prochiral ketimines so that synthetically useful biocatalysts can be identified. There is also scope to apply dynamic combinatorial screens to other reaction classes and thus obtain biocatalysts for a range of reactions that are not feasible at present.

4. Experimental

4.1. Microorganisms and growth

A. woodii DSM 1030 was maintained and grown in 'Balch' medium as described previously[14] at 30 °C in an anaerobic cabinet (Don Whitley). The medium was made anaerobic as follows. The basal medium solution containing NH_4Cl (0.1 g l^{-1}), $MgSO_4.7H_2O$ (0.1 g l^{-1}) K_2HPO_4 (0.4 g l^{-1}), KH_2PO_4 (0.4 g l^{-1}), Cysteine HCl (0.5 g l^{-1}), yeast extract (2.0 g l^{-1}), resazurin solution (1.0 ml l^{-1}; 0.1 g l^{-1}), $CaCO_3$ (for agar plates only; 5 g l^{-1}) was boiled and then cooled in an ice bath whilst gassing with N_2. The anaerobic solution was transferred by N_2 pressure to a Buchner flask (containing agar powder if required; 20 g l^{-1}) fitted with a football bladder on the side arm, which had been evacuated and flushed with N_2. The flask was sealed and autoclaved at 121 °C for 15 min. The football bladder acted as a buffer for pressure changes during autoclaving. The flask was cooled for approximately 40 min in a water bath at 60 °C, and transferred to the anaerobic cabinet. All other medium components were made anaerobic by dispensing into universal bottles, sealing with Suba seals and sparging with O_2-free nitrogen for 20 min via syringe needles inserted through the Suba seals. Wolin B trace elements, sodium sulfide, selenite-tungstate and caffeate solutions were then autoclaved at 121 °C for 15 min and transferred to the anaerobic cabinet when cool, whilst Wolin B vitamins, $NaHCO_3$ solution and fructose solution (100 g l^{-1}) were transferred to the anaerobic cabinet and filter sterilized (Acrodisc 32, pore size 0.2 μm).

The complete medium was prepared by adding deoxygenated, sterilised solutions to the basal medium in the following sequence, with thorough mixing after addition of each solution: Wolin B vitamin solution (10 ml l^{-1}), Wolin B trace elements (10 ml l^{-1}), $NaHCO_3$ solution (20 ml l^{-1}), $NaS.9H_2O$ (10 ml l^{-1}), selenite–tungstate solution (2 ml l^{-1}), fructose solution (as required). The medium was then transferred to sterile culture vessels in the anaerobic cabinet.

Stock cultures were maintained on agar plates and subcultured every 2 weeks and were grown with fructose (2.5 g l^{-1}) and $CaCO_3$. All other cultures were grown with fructose at 5 g l^{-1}, without agar and $CaCO_3$. Sodium caffeate (1 mM) was added when required and was prepared by dissolving caffeic acid in 10% of the final volume in deoxygenated NaOH (1 M) in the anaerobic cabinet. This was diluted to 60% of the final volume with deoxygenated H_2O, adjusted to pH 7.0 using deoxygenated HCl (1 M) and then made to the desired volume by adding deoxygenated H_2O to prepare a 0.15 M solution. The NaOH, water and HCl had been deoxygenated by sparging with N_2 for 20 min. The solution was filter sterilised, kept under anaerobic conditions and used within 2 d.

Precultures (15 ml) were inoculated with a single colony from a stock culture and grown for 48 h. Inocula for biocatalyst production (15 ml) were inoculated from the precultures (10% v/v) and incubated for 48 h. Two cultures were then used to inoculate 400 ml medium in Duran bottles capped with foam stoppers. The cultures were mixed on a

magnetic stirrer at 100 rpm. The empty Duran bottles had been autoclaved aerobically. Air was removed using the air lock of the anaerobic cabinet, before moving the bottles into the anaerobic cabinet and dispensing the medium into the bottles.

4.2. Cell harvesting

Caffeate-containing cultures were harvested after caffeate in the medium had been completely reduced. Cultures grown without caffeate had been inoculated at the same time as the caffeate-containing cultures and were also harvested at the same time. The cultures were transferred to airtight, screw-capped bottles (COWIE PTFE Technology, UK). The bottles were sealed, removed from the anaerobic cabinet and centrifuged at 5000 rpm for 30 min in a Thermo IEC Centra-3M Centrifuge (USA). The supernatants were discarded and the cells washed twice using deoxygenated 20 mM potassium phosphate buffer (pH 7.0). The buffer was deoxygenated by the same method as described for the basal medium. The cells were then resuspended in the buffer at approximately 10% of original culture volume and used as the biocatalyst.

4.3. Preparation of reaction mixtures

Virtual libraries were prepared from butylamine, aniline, butanal and benzaldehyde which were stored under N_2. Stock solutions (0.1 M) were dissolved in deoxygenated distilled water (butylamine, aniline, butanal) or tetradecane (benzaldehyde) in a fume cupboard under N_2 flow and then transferred to the anaerobic cabinet to prepare the reaction mixtures. The solvents were deoxygenated by sparging with N_2 for 20 min.

The overall volume of the reaction mixtures was 6.25 ml in 20 ml bottles, and all substrates were added to 2.5 mM relative to the overall volume. The aqueous phase (5 ml) contained harvested cell suspension (1 ml) mixed with the butylamine, aniline, and butanal solutions in potassium phosphate buffer (pH 7.0). The overall buffer concentration was adjusted to 20 mM by adding a 40 mM solution to compensate for the volume of aqueous substrate solutions added and then making the final aqueous phase volume to 5 ml with a 20 mM solution. The organic phase (1.25 ml) contained benzaldehyde solution mixed with anaerobic tetradecane. Thus, the phase ratio was 0.2.

The reaction bottles were sealed with suba seals, which were secured with self-lock cable ties. Reactions were started by adding hydrogen as the electron donor. A slight vacuum was created by removing the headspace gas (approximately 13.75 ml) into a 50 ml syringe, and then a slight over-pressure was created by injecting H_2 (50 ml). The reaction mixtures were stirred using a magnetic stirrer bar at 300 rpm for 24 h. Samples of the reaction mixture were placed in a narrow neck test tube and the phases allowed to separate. Samples from the tetradecane phase and the aqueous phase were transferred into Eppendorf tubes and centrifuged at 13,000 rpm for 5 min using a microcentrifuge (MICRO 20, CE, Germany) to remove the cells from the samples and remove residual water from the tetradecane phase. The supernatants were transferred into GC autosampler glass vials and stored at $-20\,^{\circ}C$ for subsequent analysis by GC–MS.

When caffeate was used as the substrate, the same reaction mixture was used except that only caffeate (2.5 mM) was added as the sole substrate. Samples (0.2 ml) were taken at intervals to measure rates of caffeate reduction. Samples were made aerobic and put on ice to stop the reaction, then centrifuged at 13,000 rpm for 3 min. The aqueous phase was withdrawn and diluted 40 times for determination of residual caffeate.

4.4. Analytical methods

Cell growth was estimated by measuring the OD at 660 nm after an appropriate serial dilution of samples of cultures. Growth rates (μ) were calculated from plots of ln OD (x) versus time (t), using the equation $\ln x_t = \mu t + \ln x_0$. Biocatalyst concentrations were determined by converting OD_{660} to dry weight using a calibration curve. This was prepared using a harvested cell preparation, which had been washed and resuspended in distilled water in place of phosphate buffer and then diluted to give a range of OD values. Dry weights were determined by centrifuging the diluted samples, washing the pellets into preweighed glass vessels using distilled water and drying at 105 °C overnight. An OD value of 1 was equivalent to a dry weight of $0.382\ g\ l^{-1}$.

Reaction products from dynamic combinatorial screens were identified by GCMS analysis using a Hewlett–Packard 5890A series 1 gas chromatograph coupled to a VG Tritech TS250E mass spectrometer. Ultra-high purity helium (99.9995%) was used as the carrier gas at 120 kP head pressure. The samples from the tetradecane phase were analysed directly by on-column injection of samples (0.25 μl) at 140 °C onto a Durabond DBwax capillary column (30 m×0.25 mm, 0.25 μm phase thickness, Jones Chromatography). Sample components were resolved using a temperature programme comprising isothermal chromatography at 140 °C for 2 min, followed by a linear gradient of $15\ ^{\circ}C\ min^{-1}$ to produce a final temperature of 250 °C, which was maintained for 5 min.

The aqueous phase samples were also analysed without extraction, by on-column injection of samples (0.5 μl) at 120 °C onto a Durabond DB1701 capillary column (30 m×0.25 mm, 0.25 μm phase thickness, Jones Chromatography). Sample components were resolved using a temperature programme comprising isothermal chromatography at 120 °C for 2 min, followed by a linear gradient of $15\ ^{\circ}C\ min^{-1}$ to produce a final temperature of 265 °C which was maintained for 5 min.

Mass spectra were acquired using positive ion electron impact ionization at 70 eV with the source temperature at 240 °C. Mass spectra were scanned from 450 to 45 mass units over 1 s and the resolution was 300. Analyses were conducted at the maximum sensitivity of the instrument. Compounds were identified by comparing mass spectra with authentic spectra from the National Institute of Standards (NIST) database, or by comparing the spectra and retention times with authentic standards when these were available.

Caffeate concentrations were determined by measuring absorbance at 312 nm using a UV spectrophotometer (S&M CECIL 1020, UK) and calculated according to a reference curve of absorbance against caffeate concentration. Reduction of caffeate was determined by measuring the loss of absorbance at 312 nm due to hydrogenation of the C=C double bond.

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