Typical results are illustrated in Figure 2 for D_2O solutions. The proton spectra in Figure 2a for a 10 mM solution of Me_4NCl show the HOD peak and the methyl proton peak at various currents. The constancy of the HOD peak amplitude for currents up to 0.8 mA indicates that electro-osmosis is negligible in a properly coated cell.[10] However, above 0.9 mA with $t_f = 0.75$ s additional attenuation, probably resulting from heat induced convention, becomes evident. Figure 2b shows proton spectra for a solution 5 mM in both Me_4NCl and Et_4NCl. Spin splitting reduces the intensity of the Et_4N^+ signals, and J modulation complicates the interpretation of the methylene intensities. However, the methyl signal for Me_4N^+ and the center line of the methyl signal for Et_4N^+ provide sufficient information for the determination of corresponding mobilities. A fit of methyl intensities versus I (Figure 2a) to a cosinusoidal function yields $\mu = 3.6 \times 10^{-4}$ cm^2 V^{-1} s^{-1} which can be compared with the zero concentration result, 3.79×10^{-4} cm^2 V^{-1} s^{-1}, from conductivity measurements.[11] For the mixture we obtain 3.9×10^{-4} cm^2 V^{-1} s^{-1} and 2.5×10^{-4} cm^2 V^{-1} s^{-1} for the methyl and ethyl ammonium ions, respectively.

The potential of this method is obvious from Figure 2. The sensitivity can be improved by the use of multiple capillary tubes and higher frequency spectrometers; however, the necessity of long T_2's remains a limitation. The technique is especially promising for small ions and for microemulsions. A two-dimensional version of the experiment, based on Fourier transformations with respect to both t_f and t_a, has also been developed for the study of velocity distributions. Applications will be published elsewhere.

Acknowledgment. This work was supported under National Science Foundation Grant no. CHE-8617029.

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Phenoxazinone Synthase: Enzymatic Catalysis of an Aminophenol Oxidative Cascade

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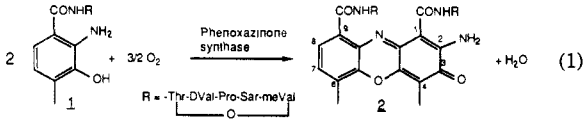
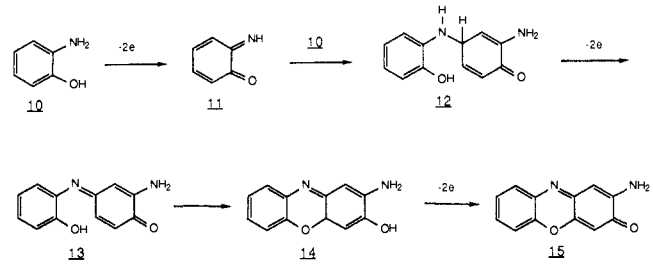
Phenoxazinone synthase catalyzes the oxidative coupling of two molecules of an aminophenol to form the phenoxazinone chromophore.[1] This reaction constitutes the final step in the biosynthesis of actinomycin **2**[2] and is a complex six-electron oxidative condensation (eq 1). The mechanism of the enzymatic reaction has not been elucidated and was of interest to us because of the novelty of the chemistry involved.



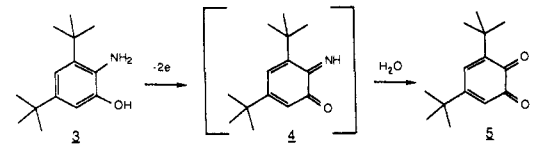
(1)

Phenoxazinone synthase has been cloned and overproduced in *Streptomyces lividans*[3] and can be readily isolated in 100-mg quantities.[4] The subunit molecular weight is 88 000 daltons. In its active form the enyzme consists primarily of a mixture of dimers and hexamers.[4] The enzyme is a copper-containing protein[5] and catalyzes the oxidation of a wide variety of aminophenols to phenoxazinones.[2]

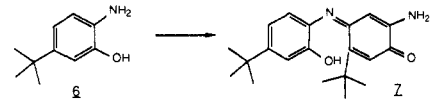
Scheme I



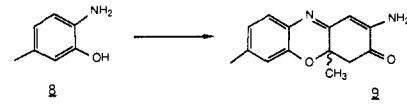
By using a variety of substituted aminophenols we have been able to block the synthesis of phenoxazinone at various intermediate stages.[6] Enzyme-catalyzed oxidation of 3,5-di-*tert*-butyl-2-aminophenol (**3**) generated the *o*-quinone **5**[7] (eq 2). This



(2)



(3)



(4)

product presumably arose from hydrolysis of the intermediate *o*-quinoneimine **4** which was too sterically hindered to undergo subsequent conjugate addition. Enzyme-catalyzed oxidation of 5-*tert*-butyl-2-aminophenol (**6**)[8] resulted in the formation of quinone-anil **7** in which the bulky *tert*-butyl group blocked the second conjugate addition (eq 3). Enzyme-catalyzed oxidation of 5-methyl-2-aminophenol (**8**) produced the dihydrophenoxazinone **9** in which the methyl group at C4a blocked the final dehydrogenation (eq 4). These results are incorporated into the mechanistic proposal shown in Scheme I.

Quinoneimine **11** reacts rapidly with aminophenol to form phenoxazinone.[9] This observation prompted us to determine if intermediates are released from the enzyme prior to phenoxazinone formation. Dihydrophenoxazinone **16**[10] was prepared and found to be instantaneously oxidized to phenoxazinone by air. To determine if the second conjugate addition occurred at the active site, the chirality at C4a of **9** was examined. Racemic **9** was

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(4) Choy, H.; Jones, G. *Arch. Biochem. Biophys.* **1981**, *211*, 55. Due to the high level of enzyme in the overproducing strain, we have been able to simplify the isolation procedure. Hydroxyapatite chromatography of the protein fraction precipitated by 35-45% ammonium sulfate resulted in pure enzyme.
(5) Samples were analyzed for iron, manganese, copper, molybdenum, cobalt, chromium, and nickel by inductively coupled plasma emission spectroscopy. The enzyme as isolated contained 0.5-2.0 coppers/subunit. However, addition of exogenous copper increased the activity of the preparation. The copper content of the reconstituted system is 5-7 coppers/subunit.
(6) Enzyme assays were run in sodium acetate buffer (3 mL, 100 mM, pH 5) containing 2-5 mM substrate (added from a freshly prepared stock solution in DMSO) and 5 μg of enzyme. Product formation was monitored spectrophotometrically. For product isolation, 5 mL of the assay mixture was treated with 100 μg of enzyme at 37 °C for 20 min. The reaction mixture was then extracted, and the product was purified by chromatography.
(7) This product was identified by NMR and MS comparison with literature spectra. Harmalker, S. P.; Sawyer, D. T. *J. Org. Chem.* **1984**, *49*, 3579.
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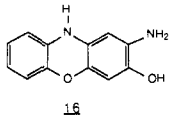

16

prepared by ferricyanide oxidation of 5-methyl-2-aminophenol (**8**).[10] The diastereomeric methyl groups of the Mosher amide of **9** were clearly distinguishable by NMR.[11] Analysis of the enzymatic product demonstrated that the configuration at C4a was racemic.[12] These observations suggest that an intermediate dissociates from the enzyme prior to the second conjugate addition and that subsequent steps do not occur with enzymatic catalysis.

The catalytic requirements for phenoxazinone formation can be greatly simplified if we propose that the aminophenol functionality is regenerated after each conjugate addition by a rapid tautomerization. In this way, what initially appears to be a complex reaction can be reduced to a sequence of three consecutive two-electron aminophenol oxidations. This sequence represents an enzyme-catalyzed oxidative cascade with the rate of oxidation increasing as the aminophenol becomes progressively more electron rich, thus allowing for nonenzymatic steps toward the end of the sequence. The mechanistic details of how the active site copper catalyzes the transfer of electrons from the aminophenol to oxygen are currently under investigation.

Acknowledgment. We thank George H. Jones (University of Michigan) for providing us with the overproducing strain and for helpful advice on how to grow it. We gratefully acknowledge financial support of this research in part by Cornell Biotechnology, the Dreyfus Foundation, Merck, Sharp and Dohme, and the National Institute of Health (CA45251-01).

Supplementary Material Available: Listing of spectral data for **6**, **7**, **9**, and **16** (1 page). Ordering information is given on any current masthead page.

(11) This was prepared by treating **9** with the Mosher acid and DCC in dichloromethane: ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.25 (s, 1.5 H), 1.28 (s, 1.5 H), 2.33 (s, 3 H), 3.1 (d, J = 11 Hz, 1 H), 3.18 (d, J = 11 Hz, 1 H), 3.49 (s, 3 H), 6.71 (s, 1 H), 6.83 (d, J = 8 Hz, 1 H), 7.3 (d, J = 9 Hz, 1 H), 7.43 (ArH, 3 H), 7.53 (ArH, 2 H), 8.21 (s, 1 H), 9.4 (d, J = 9 Hz, 1 H); MS, 458 (96), 459 (45%), 269 (41), 189 (100).

(12) When **9** was treated with deuteriated assay buffer at 37 °C for 2 h no deuterium incorporation was detected, thus precluding the possibility of facile racemization of **9** during its isolation.

Cyclopentenones from the Reaction of Alkynes with Cyclopropylcarbene–Chromium Complexes

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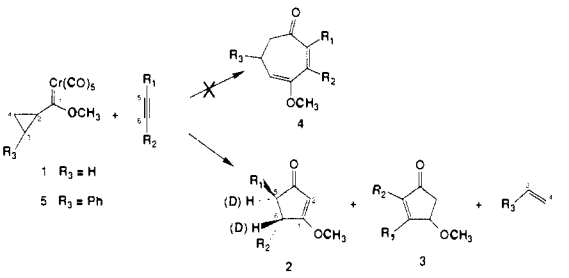
As part of a program directed at developing approaches to the synthesis of odd-membered ring size systems employing cycloaddition reactions,[1] we have investigated the reaction of the cyclopropylcarbene–chromium complex **1**[2] with alkynes[3] (Scheme I). Recently, it has been shown that the reaction between alkynes and α,β-unsaturated carbene–chromium complexes produces aromatic rings.[3a] We anticipated that the analogous reaction, which employs cyclopropyl-substituted carbene–chromium complexes and alkynes, would produce cycloheptadienone derivatives such as compound **4**. However, the reaction of cyclopropylcarbene complex **1** and alkynes did not produce cycloheptadienone **4**, rather it gave exclusively the cyclopentenone derivatives **2** and **3**, plus an alkene fragment. This reaction was found to be general for a variety of alkynes. We herein report our preliminary studies of this remarkable five-membered ring-forming reaction.

Scheme I[a]



[a] Table entry letters define R_1 and R_2.

Table I. Reaction of Complex **1** with Alkynes in Refluxing Aqueous Dioxane[a,b]

entry[c]	R_1	R_2	yield 2 (%)	trans/cis 2	yield 3 (%)
A	Ph	Ph	79	24:1	4
B	Ph	H	62		0
C	Ph	CH_3	73	9:1	12
D	n-C_3H_7	n-C_3H_7	55	7:1	0
E	n-C_3H_7	H	68		0
F	$-(CH_2)_4OH$	H	54		0
G	$-(CH_2)_4OSi(t\text{-Bu})Me_2$	H	58		0
H	cis-CH=CHOCH$_3$	H	42[d]		0
I	-COOEt	H	0		0

[a] In all cases, the alkyne (0.1 M in dioxane) was added to a refluxing solution of complex **1** in aqueous dioxane via syringe pump over a period of 4–6 h. [b] All compounds were fully characterized (see Supplementary Material). [c] Entry letters define R_1 and R_2 for compounds **2** and **3**. [d] The product was obtained as a 3:2 trans/cis mixture about the enol ether double bond.

When complex **1** was allowed to react with diphenylacetylene (1.0 M solution of both components in THF) at 65 °C under nitrogen, an intractable reaction mixture was obtained. Presumably, this was due to polymerization of the alkyne.[4,5] When diphenylacetylene was slowly added to a solution of complex **1** in THF, a new compound, **2A** (R_1, R_2 = Ph),[6] was obtained in 34% yield. A minor product, **3A**, was obtained in 6% yield. Compound **2A** was formed exclusively (41%) when the reaction was performed in refluxing dioxane. The most reasonable correlation between compound **2A** and the reactants is outlined in Scheme I. The carbonyl carbon arises from a CO ligand of the carbene complex, and carbons 1 and 2 of cyclopentenone **2A** come from carbons 1 and 2 of the carbene complex. Carbons 5 and 6 correspond to the alkyne carbons of diphenylacetylene. This

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(5) The polymerization can be suppressed if the concentration of alkyne is kept low. Wulff, W. D.; Kaesler, R. W.; Peterson, G. A.; Tang, P.-C. *J. Am. Chem. Soc.* **1985**, *107*, 1060–1062.

(6) Spectral data for **2A**: ^{1}H NMR ($CDCl_3$) δ 7.2 (m, 6 H), 7.0 (m, 4 H), 5.58 (d, 1 H, J = 1.1 Hz), 3.98 (dd, 1 H, J = 3.3, 1.1 Hz), 3.83 (s, 3 H), 3.59 (d, 1 H, J = 3.3 Hz); ^{13}C NMR ($CDCl_3$) δ 203.7 (s), 190.0 (s), 139.3 (s), 138.8 (s), 128.9, 128.7, 127.6, 127.4, 127.2, 127.0 (overlapping in SFORD spectrum), 104.5 (d), 62.4 (d), 59.0 (q), 56.4 (d); IR ($CDCl_3$) 3040 (m), 2945 (m), 1694 (s), 1598 (s), 1500 (m), 1457 (m), 1443 (m), 1358 (s), 1347 (s), 1170 (s) cm^{-1}; MS, (El), 264 (parent), 233, 205, 187, 159, 128, 115, 102, 91, 69; high resolution MS; calcd for $C_{18}H_{16}O_2$ 264.1150, observed 264.1150. Spectral data for **3A**: ^{1}H NMR ($CDCl_3$) δ 7.15–7.36 (m, 10 H), 5.04 (dd, 1 H, J = 5.9, 2.0 Hz), 3.39 (s, 3 H), 2.96 (dd, 1 H, J = 18.3, 5.9 Hz), 2.66 (dd, 1 H, J = 18.3, 2.0 Hz); IR ($CDCl_3$) 3060 (m), 2930 (m), 1705 (s), 1600 (m), 1440 (m), 1350 (m), 1260 (m), 1210 (s), 1180 (s) cm^{-1}. Compound **3A** rearranges to **2A** upon treatment with sodium methoxide/methanol.

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