J|A|C|S COMMUNICATIONS

Published on Web 07/07/2009

Revisiting the IspH Catalytic System in the Deoxyxylulose Phosphate Pathway: Achieving High Activity

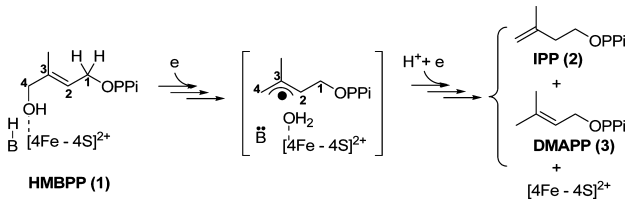
Youli Xiao,[†] Lisa Chu,[†] Yiannis Sanakis,[‡] and Pinghua Liu*,[†]

Department of Chemistry, Boston University, Boston, Massachusetts 02215, and Institute of Materials Science, NCSR "Demokritos", 15310 Ag. Paraskevi, Attiki, Greece

Received May 13, 2009; E-mail: pinghua@bu.edu

As one of the largest classes of natural products, isoprenoids are characterized by their structural diversity. Starting from two C_5 isoprene building blocks, isopentenyl diphosphate (IPP, **2**) and its isomer dimethylallyl diphosphate (DMAPP, **3**, Scheme 1), plants alone produce more than 30 000 isoprenoids.[1,2] For several decades, it was believed that the mevalonate (MVA) pathway elucidated by Bloch, Cornforth, and Lynen was the only pathway for IPP and DMAPP biosynthesis.[1,3,4] Based on the pioneering work from Arigoni and Rhomer's laboratories, the collaborative efforts from several laboratories in the past decade led to the identification of genes and intermediates involved in a second isoprenoid biosynthetic pathway, the deoxyxylulose phosphate (DXP) pathway.[5−11] The DXP pathway is found in most eubacteria, green algae, and chloroplasts of higher plants as well as the malaria parasite *Plasmodium faciparum*, whereas the MVA pathway is present in animals, fungi, and archaebacteria.[7] The end product of the MVA pathway is IPP, which is isomerized to DMAPP by isopentenyl diphosphate isomerase.[12−15] In contrast, the IspH enzyme in the DXP pathway catalyzes the formation of both IPP and DMAPP. To date, IspH activities have been measured under many different conditions, and in most cases, the activity is low. In this work, on the basis of a systematic examiniation of the effect of redox dyes with different redox potentials on IspH activity, an *Escherichia coli* IspH specific activity of 30.4 μmol min^{-1} mg^{-1} (k_{cat} of 1125 min^{-1}) was achieved; this is 97-fold higher than that for the previously reported NADPH-flavodoxin reductase (Fpr)−flavodoxin (FldA) reducing system.[16−18]

Scheme 1. IspH Reaction



IspH in the DXP pathway catalyzes the reductive dehydration of (*E*)-4-hydroxy-3-methyl-2-butenyl diphosphate (HMBPP, **1**) to form both **2** and **3**.[16,17,19−26] IspH is a protein containing an iron−sulfur cluster, and this cluster plays a critical role in the catalysis.[17,21,24,27] The *E. coli* IspH, a 36 kDa protein, has three conserved Cys residues (C12, C96, and C197). Replacing any of the three conserved cysteine residues reduced the catalytic activity by a factor of more than 70 000.[17] The IspH iron−sulfur cluster has been assembled in vitro by iron and sulfur reconstitution[24,28] and in vivo by coexpression with the *E. coli isc* operon,[17] which is responsible for the assembly and insertion of iron−sulfur clusters into iron−sulfur cluster-containing proteins.[29]

Recent EPR studies suggested that the isolated IspH, which initially contains a [4Fe−4S]$^{2+}$ cluster,[24] can lose its unique iron-site iron during purification to form a [3Fe−4S]$^{+}$ cluster.[17] Reduction of the iron−sulfur cluster of reconstituted IspH using a 50-fold excess of dithionite (DT) relative to IspH resulted in the formation of [4Fe−4S]$^{+}$, which is believed to be the catalytic species in the IspH-catalyzed reactions.[24]

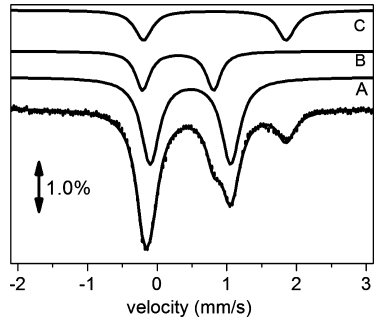


Figure 1. Mössbauer spectrum from isolated IspH at 4.2 K with an external magnetic field of 0.1 T applied perpendicular to the γ-ray beam. Solid lines are theoretical simulations assuming three different doublets, as described in Table 1.

Table 1. Mössbauer Parameters for Simulating the Spectrum in Figure 1

doublet	δ (mm/s)[a]	ΔE_Q (mm/s)[b]	area (%)[c,d]
A	0.48	1.16	53.4
B	0.30	1.02	19.0
C	0.83	2.04	17.6

[a] Uncertainty of ±0.02 mm/s. [b] Uncertainty of ±0.03 mm/s. [c] Uncertainty of ±5%. [d] The remaining 10% is most likely from a [3Fe−4S]$^{+}$ species (see the SI).

To provide further evidence to support the role of the [4Fe−4S] cluster in IspH-catalyzed reactions, we overexpressed and purified ^{57}Fe-labeled strep-tagged IspH using streptavidin resin (IBA Inc.) using a slight modification of our previously reported IspH purification protocol [see the Supporting Information (SI)].[16] Iron and sulfur analyses using the methods reported by Fish[30] and Beinert[31] indicated that the isolated IspH has 3.7 iron ions and 3.8 sulfur atoms per IspH monomer. The isolated ^{57}Fe-loaded IspH was then characterized using Mössbauer spectroscopy. Figure 1 shows the Mössbauer spectrum at 4.2 K with a 0.1 T magnetic field applied perpendicular to the γ-ray beam. The spectrum was simulated with three quadruple doublets with the parameters listed in Table 1. The parameters of doublet A, which accounts for ~53% of the total iron, are consistent with a [4Fe−4S]$^{2+}$ cluster. The isomer shift of doublet B is consistent with a tetrahedral high-spin ferric site comprising sulfur ligands. The behavior of B as a function of temperature and magnetic field is consistent with either a diamagnetic species or a species with an integer spin. The value of quadruple splitting for doublet B is too large to be attributed to a

[†] Boston University.
[‡] NCSR "Demokritos".

$[2Fe-2S]^{2+}$ cluster or the ferric site of a $[3Fe-4S]^{0}$ one.[32,33] The parameters of doublet C are more consistent with a tetrahedral high-spin ferrous site. The assignment of doublets B and C is not clear at the moment. Interestingly, the A/B and A/C ratios are close to 3:1, the value expected for a $[4Fe-4S]^{2+}$ cluster with a unique iron site (having either localized ferric or ferrous character). Such differentiated sites have been observed in Fe−S enzymes and synthetic clusters containing the $[4Fe-4S]^{2+}$ core.[34,35] A definite determination of the nature of doublets B and C requires more experiments, which are in progress. More detailed analysis also indicates the presence of roughly 10% $[3Fe-4S]^{+}$ species, which is consistent with the EPR results (see the SI).[24] In conclusion, the present Mössbauer spectroscopic results indicate that in the isolated ^{57}Fe-loaded IspH, at least 53% of the total iron is found in an $[4Fe-4S]^{2+}$ form, verifying earlier studies that invoked the presence of an Fe−S cluster in IspH.

Surprisingly, when ^{57}Fe-loaded IspH was incubated with NADPH-Fpr−FldA, which is proposed to be the in vivo IspH reducing system,[17,25] no noticeable difference in the Mössbauer spectrum was observed, and almost the same IspH Mössbauer spectrum was obtained with or without NADPH-Fpr−FldA (see the SI). Because the IspH-catalyzed reaction is a reductive dehydration process, the $[4Fe-4S]^{+}$ is proposed to be the catalytic species.[24] Our results suggested that under the current conditions, when IspH is reduced by the NADPH-Fpr−FldA system from the $[4Fe-4S]^{2+}$ state to the $[4Fe-4S]^{+}$ state, the $[4Fe-4S]^{+}$ cluster is present in only a very small portion. This is consistent with the relatively low activity when NADPH-Fpr−FldA is used as the reduction system and the wide range of IspH activities under different conditions reported in the literature (see Table 3).

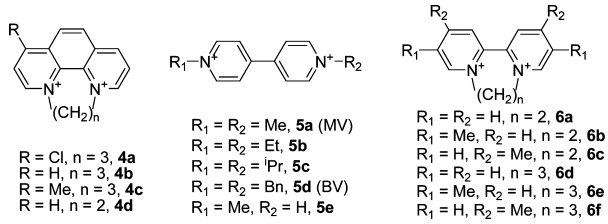


Figure 2. Redox mediators used in the IspH activity assay.

Table 2. Catalytic Activities of IspH Using Redox Mediators with Different Redox Potentials[a]

entry	mediator	conversion (%)[b]	activity (min^{-1})[c]	$E°$ (mV vs NHE)[d]
1	**4a**	<1	n.d.	−110[36]
2	**4b**	<1	n.d.	−130[37]
3	**4c**	<1	n.d.	−220[36]
4	**4d**	<1	n.d.	−250[37]
5	**6a**	20	133	−312[38]
6[e]	**5d**	∼5	33	−360[37]
7	**6b**	40	266	−442[38]
8	**5a**	50	333	−446[39]
9	**6c**	60	400	−450[38]
10	**5b**	41	273	−480[40]
11	**5c**	24	160	−495[40]
12	**6d**	10	67	−510[38]
13	**6e**	∼5	33	−625[38]
14	**6f**	30	200	−649[38]
15	**5e**	<1	n.d.	−720[41]
16	NADPH-FldA−Fpr		6.67[f]	

[a] Reactions were performed in 100 mM Tris-HCl (pH 8.0) containing 1.0 mM HMBPP, 50 nM IspH, 5.0 mM DT, and 1.0 mM mediator at 37 °C for 30 min. [b] Estimated on the basis of the integration of the 400 MHz ^{1}H NMR signals from the methyl groups in the products and substrate (see the SI). [c] Calculated from end-point conversion. [d] Superscripts give the references from which the data were obtained. [e] The assay was performed at pH 6.5 to avoid the precipitation that occurs at higher pH. [f] Data from ref 16.

Table 3. Reported IspH Activities under Different Conditions

source[ref]	reductant system	A_{410}/A_{280}	activity (μmol min^{-1} mg^{-1})	K_m (μM)
E. coli[21]	NADPH-Fpr−FldA[a]	0.12[b]	0.003[c]	n.d.
	DAF[d]	0.12[b]	0.4[c]	n.d.
E. coli[24]	NADPH-Fpr−FldA[a]	0.14[e]	0.002[f]	n.d.
	DAF[d]	0.14[e]	0.011[f]	n.d.
	NADPH-Fpr−FldA[a]	0.53[e,g]	0.044[f]	n.d.
	DAF[d]	0.53[e,g]	0.030[f]	n.d.
E. coli[17]	NADPH-Fpr−FldA[a]	0.38[i]	0.38[c]	n.d.
	NADPH-Fpr−FldA[a,h]	0.38[i]	0.7,[c] 0.66,[j] 0.55[k]	30
	NADH-Fpr−FldA[l,h]	0.38[i]	0.49[c]	n.d.
	NADPH-Fdr−FldA[m,h]	0.38[i]	0.52[c]	n.d.
	NADPH-Fdr−Fdx[n,h]	0.38[i]	0.032[c]	n.d.
	DAF[d]	0.38[i]	3.4[c]	n.d.
	DT[o]	0.38[i]	$<1.5 \times 10^{-5}$[c] or 0	n.d.
E. coli[16]	NADPH-Fpr−FldA[a]	0.40[p]	0.31[k]	<15
E. coli (this work)	DT−MV	0.40	16.3	19.7
	DT−MDQ	0.40	30.4	31.6
A. aeolicus[28]	DT−MV[q,r]	n.d.	6.6[s]	590
P. falciparum[44]	DT−MV[q]	n.d.	2.1[s]	39
	NADPH-Fd−FNR[t]	n.d.	10.3[k]	n.d.

[a−t] Detailed reaction conditions are listed in the SI.

To examine whether the redox potential difference of different redox systems reported in literature is the reason for the wide range of reported IspH activities, three series of diquaternary salts, involving 1,10-phenanthroline (**4a**−**d**), 4,4′-bipyridine (**5a**−**e**), and 2,2′-bipyridine (**6a**−**f**), were either obtained from commercial sources (**5a**, **5d**) or synthesized chemically in our laboratory according to reported literature procedures (Figure 2; also see the SI).[42] These redox mediators have a broad range of redox potentials, from −100 to −720 mV (vs NHE).[43]

Once these redox mediators were obtained, they were used as mediators to transfer electrons from an artificial reductant, DT, to the IspH iron−sulfur cluster. The redox potential for DT is −600 mV at pH 8.0.[39] When IspH activities were assayed by direct ^{1}H NMR monitoring of its product formation, as reported in our previous studies,[16,26] a clear trend was observed (Table 2). When the redox potential ($E°$) of the redox mediator is higher than −250 mV, there is almost no detectable IPP or DMAPP formation (Table 2, entries 1−4). When the redox potential of the redox mediator is further decreased, the IspH catalytic activity increases (Table 2, entries 5−9); the highest activity (∼400 min^{-1}) was observed for the redox mediator 6,7-dihydro-2,11-dimethyldipyrido[1,2-*a*:2,1-*c*]pyrazinium dibromide (MDQ, **6c**) ($E° = -450$ mV). When the redox potential of the redox mediator is further decreased, the IspH activity begins to decrease again (Table 2, entries 10−15).

The highest activity was found when MDQ (**6c**) was used as the redox mediator; this activity was at least 60-fold higher than that using NADPH-Fpr−FldA as the reducing system, as determined by the ^{1}H NMR assay (Table 2, entries 9 and 16). The ^{1}H NMR assay was an end-point assay, and the substrate conversion was analyzed after 30 min of reaction. To confirm the high activity result, kinetic analyses were carried out using reduced methyl viologen (MV, **5a**; $\varepsilon_{734} = 2.665$ mM^{-1} cm^{-1}, $\varepsilon_{604} = 13.600$ mM^{-1} cm^{-1})[39] and MDQ ($\varepsilon_{760} = 2.90$ mM^{-1} cm^{-1})[37] as the reductant. The kinetic analysis results (k_{cat} = 604 min^{-1} and $K_m = 19.7 \pm 2.4$ μM using MV; $k_{cat} = 1125$ min^{-1} and $K_m = 31.6 \pm 2.2$ μM using MDQ) are consistent with the end-point ^{1}H NMR assay results. The K_m values for HMBPP are close to that reported earlier using NADPH-Fpr−FldA as the reducing system. However, the k_{cat} values for IspH are 52- and 97-fold larger with the use of reduced MV and MDQ, respectively, as the reductant (see the

SI). The IPP and DMAPP produced were also isolated and characterized (see the SI).[16]

From experiments using IspH from various sources along with iron−sulfur clusters assembled by either in vitro iron−sulfur reconstitution or in vivo *isc* operon-mediated maturation, a wide range of activities (2.0 nmol min^{-1} mg^{-1} to 10.3 μmol min^{-1} mg^{-1}) have been reported in the literature in the past decade (Table 3). The reduction systems used include NAD(P)H-FldA−Fpr, NADPH-ferredoxin (Fdx)−ferredoxin reductase (Fdr), photoreduced deazaflavin (DAF), and DT alone or with MV as the electron shuttle. In the literature, the highest reported activity was for IspH from the malaria parasite *Plasmodium falciparum* with ferredoxin (Fd) and ferredoxin-$NADP^+$ reductase (FNR) from the same organism as the reduction system and NADPH as the electron source.[44] For *E. coli* IspH, Gräwert et al.[17] reported an activity of 0.7 μmol min^{-1} mg^{-1} at 37 °C using NADPH-Fpr−FldA as the reduction system. Using photoreduced deazaflavin as the reductant, they reported an activity of 3.4 μmol min^{-1} mg^{-1}. However, they could not detect enzymatic activity using DT as the reducing agent.

For such a wide range of activities reported in the literature, the activity assays were carried out under different conditions. At least three parameters were different for the various studies: the organism from which the IspH was obtained, the way the iron−sulfur cluster was incorporated, and the redox system used to reduce the IspH. The widely different conditions used in those studies prevent a direct comparison of the activities and a determination of the key factor responsible for such a dramatic difference in the IspH activities. In this work, using *E. coli* IspH as the enzyme and DT as the ultimate electron source, we systematically examined the role of redox mediators in IspH activity. Our results not only indicate the importance of a proper redox mediator but also show a 97-fold improvement in the *E. coli* IspH activity relative to that for the *E. coli* NADPH-Fpr−FldA system.

The NADH-Fpr−FldA system is proposed to be the natural electron source for *E. coli* IspH.[16,17,21,25] Using an *E. coli* strain harboring the plasmid for the MVA pathway genes, Puan et al.[25] identified *fldA* as an essential gene on the basis of random transposon mutagenesis. It is believed that the reduced FldA provides electrons to reduce the $[4Fe-4S]^{2+}$ in IspG and IspH to $[4Fe-4S]^{+}$, which is then used to mediate the reductive dehydration reactions. From the results listed in Table 3, high IspH activities were obtained with redox mediators having redox potentials lower than −300 mV. The redox potentials for the FldA FMN cofactor oxidized/semiquinone pair and semiquinone/hydroquinone pair are −245 and −455 mV, respectively.[45] The redox potentials for the oxidized/semiquinone pair and the semiquinone/hydroquinone pair of the Fpr FAD cofactor are −308 and −268 mV, respectively.[46] The relatively high redox potentials of the Fpr FAD cofactor explain why there is a nearly 97-fold difference in the IspH activities using NADPH-Fpr−FldA and DT−MDQ as the reducing systems. On the basis of these results, it is tempting to suggest that IspH activity is regulated in vivo by modulation of the redox potential of its iron−sulfur cluster. Alternatively, IspH could use a redox system other than NADPH-Fpr−FldA in vivo. Our inability to reduce IspH from $[4Fe-4S]^{2+}$ to $[4Fe-4S]^{+}$ using NADPH-Fpr−FldA, as revealed by Mössbauer studies, also supports this hypothesis. These options are under investigation.

Acknowledgment. P.L. was supported by the Boston University Startup Fund and an NSF CAREER Award (CHE-0748504). We also thank the NSF-REU program (CHE-0649114) for support of L.C. (summer 2008).

Supporting Information Available: Complete author lists for refs 15, 17, 27, and 44, purification of ^{57}Fe-labeled IspH protein, Mössbauer spectrum of IspH in the presence of NADPH-Fpr-FldA, syntheses of redox dyes, steady-state kinetic IspH analysis using DT and MV or MDQ, IspH activities under different conditions, and IspH reaction product characterization. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) *Comprehensive Natural Products Chemistry: Isoprenoids Including Carotenoids and Steroids*; Cane, D. E., Ed.; Elsevier Science Ltd.: Oxford, U.K., 1999.
(2) Sacchettini, J. C.; Poulter, C. D. *Science* **1997**, *277*, 1788.
(3) Bloch, K. *Steroids* **1992**, *57*, 378.
(4) Bach, T. J. *Lipids* **1995**, *30*, 191.
(5) Phillips, M. A.; Leon, P.; Boronat, A.; Rodriguez-Concepcion, M. *Trends Plant Sci.* **2008**, *13*, 619.
(6) Proteau, P. *J. Bioorg. Chem.* **2004**, *32*, 483.
(7) Eisenreich, W.; Bacher, A.; Arigoni, D.; Rohdich, F. *Cell. Mol. Life Sci.* **2004**, *61*, 1401.
(8) Kuzuyama, T.; Seto, H. *Nat. Prod. Rep.* **2003**, *20*, 171.
(9) Rohdich, F.; Kis, K.; Bacher, A.; Eisenreich, W. *Curr. Opin. Chem. Biol.* **2001**, *5*, 535.
(10) Lichtenthaler, H. K. *Annu. Rev. Plant Phys.* **1999**, *50*, 47.
(11) Wiesner, J.; Jomaa, H. *Curr. Drug Targets* **2007**, *8*, 3.
(12) Agranoff, B. W.; Eggerer, H.; Henning, U.; Lynen, F. *J. Biol. Chem.* **1960**, *235*, 326.
(13) Ramos-Valdivia, A. C.; van der Heijden, R.; Verpoorte, R. *Nat. Prod. Rep.* **1997**, *14*, 591.
(14) Kaneda, K.; Kuzuyama, T.; Takagi, M.; Hayakawa, Y.; Seto, H. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 932.
(15) Laupitz, R.; et al. *Eur. J. Biochem.* **2004**, *271*, 2658.
(16) Xiao, Y.; Zhao, Z. K.; Liu, P. *J. Am. Chem. Soc.* **2008**, *130*, 2164.
(17) Grawert, T.; et al. *J. Am. Chem. Soc.* **2004**, *126*, 12847.
(18) Xiao, Y.; Liu, P. *Tetrahedron Lett.* **2009**, *50*, 309.
(19) Rohdich, F.; Bacher, A.; Eisenreich, W. *Bioorg. Chem.* **2004**, *32*, 292.
(20) Rohdich, F.; Hecht, S.; Gartner, K.; Adam, P.; Krieger, C.; Amslinger, S.; Arigoni, D.; Bacher, A.; Eisenreich, W. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 1158.
(21) Rohdich, F.; Zepeck, F.; Adam, P.; Hecht, S.; Kaiser, J.; Laupitz, R.; Grawert, T.; Amslinger, S.; Eisenreich, W.; Bacher, A.; Arigoni, D. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 1586.
(22) Adam, P.; Hecht, S.; Eisenreich, W.; Kaiser, J.; Grawert, T.; Arigoni, D.; Bacher, A.; Rohdich, F. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 12108.
(23) Rohdich, F.; Hecht, S.; Bacher, A.; Eisenreich, W. *Pure Appl. Chem.* **2003**, *75*, 393.
(24) Wolff, M.; Seemann, M.; Tse Sum Bui, B.; Frapart, Y.; Tritsch, D.; Garcia Estrabot, A.; Rodriguez-Concepcion, M.; Boronat, A.; Marquet, A.; Rohmer, M. *FEBS Lett.* **2003**, *541*, 115.
(25) Puan, K. J.; Wang, H.; Dairi, T.; Kuzuyama, T.; Morita, C. T. *FEBS Lett.* **2005**, *579*, 3802.
(26) Xiao, Y.; Liu, P. *Angew. Chem., Int. Ed.* **2008**, *47*, 9722.
(27) Rekittke, I.; et al. *J. Am. Chem. Soc.* **2008**, *130*, 17206.
(28) Altincicek, B.; Duin, E. C.; Reichenberg, A.; Hedderich, R.; Kollas, A. K.; Hintz, M.; Wagner, S.; Wiesner, J.; Beck, E.; Jomaa, H. *FEBS Lett.* **2002**, *532*, 437.
(29) Johnson, D. C.; Dean, D. R.; Smith, A. D.; Johnson, M. K. *Annu. Rev. Biochem.* **2005**, *74*, 247.
(30) Fish, W. W. *Methods Enzymol.* **1988**, *158*, 357.
(31) Beinert, H. *Anal. Biochem.* **1983**, *131*, 373.
(32) Weigel, J. A.; Holm, R. H.; Surerus, K. K.; Munck, E. *J. Am. Chem. Soc.* **1989**, *111*, 9246.
(33) Coucouvanis, D.; Alahmad, S. A.; Salifoglou, A.; Papaefthymiou, V.; Kostikas, A.; Simopoulos, A. *J. Am. Chem. Soc.* **1992**, *114*, 2472.
(34) Kent, T. A.; Dreyer, J. L.; Kennedy, M. C.; Huynh, B. H.; Emptage, M. H.; Beinert, H.; Munck, E. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 1096.
(35) Ciurli, S.; Carrie, M.; Weigel, J. A.; Carney, M. J.; Stack, T. D. P.; Papaefthymiou, G. C.; Holm, R. H. *J. Am. Chem. Soc.* **1990**, *112*, 2654.
(36) Miller, D.; Mclendon, G. *Inorg. Chem.* **1981**, *20*, 950.
(37) Tsukahara, K.; Wilkins, R. G. *J. Am. Chem. Soc.* **1985**, *107*, 2632.
(38) Alber, K. S.; Hahn, T. K.; Jones, M. L.; Fountain, K. R.; Van Galen, D. A. *J. Electroanal. Chem.* **1995**, *383*, 119.
(39) Mayhew, S. G. *Eur. J. Biochem.* **1978**, *85*, 535.
(40) Watt, G. D.; Burns, A.; Lough, S.; Tennent, D. L. *Biochemistry* **1980**, *19*, 4926.
(41) Guo, M.; Sulc, F.; Ribbe, M. W.; Farmer, P. J.; Burgess, B. K. *J. Am. Chem. Soc.* **2002**, *124*, 12100.
(42) Campa, C.; Camps, J.; Font, J.; Demarch, P. *J. Org. Chem.* **1987**, *52*, 521.
(43) Fultz, M. L.; Durst, R. A. *Anal. Chim. Acta* **1982**, *140*, 1.
(44) Rohrich, R. C.; et al. *FEBS Lett.* **2005**, *579*, 6433.
(45) Vetter, H., Jr.; Knappe, J. *Hoppe-Seyler's Z. Physiol. Chem.* **1971**, *352*, 433.
(46) McIver, L.; Leadbeater, C.; Campopiano, D. J.; Baxter, R. L.; Daff, S. N.; Chapman, S. K.; Munro, A. W. *Eur. J. Biochem.* **1998**, *257*, 577.

JA903778D