Siroamide: A Prosthetic Group Isolated from Sulfite Reductases in the Genus *Desulfovibrio*†

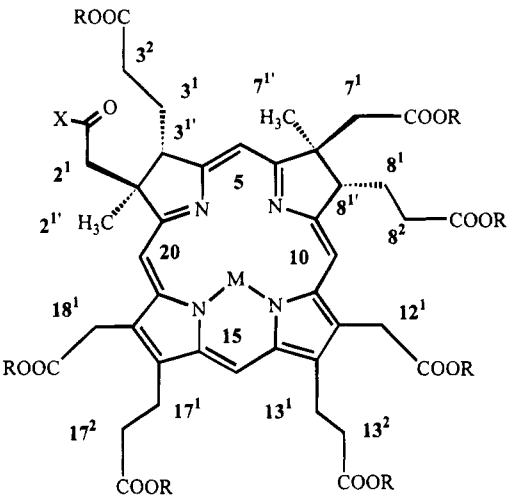
Jeffrey C. Matthews,‡ Russell Timkovich,*,‡ Ming-Yih Liu,§ and Jean Le Gall§

Department of Chemistry, University of Alabama, Tuscaloosa, Alabama 35487-0336, and Department of Biochemistry and Molecular Biology, University of Georgia, Athens, Georgia 30602

Received November 14, 1994; Revised Manuscript Received January 30, 1995⊗

ABSTRACT: While isolating siroheme from enzymes or whole cells of *Desulfovibrio* species, it was discovered that the main product after metal removal and esterification was not the octamethyl ester derivative of sirohydrochlorin, but a monoamide, heptamethyl ester derivative. The structure of this derivative was established by mass spectrometry and NMR. Nuclear Overhauser enhancement measurements in combination with chemical shift analogy arguments indicate that the 2^1-acetate has been stereospecifically amidated. Other cellular sources of siroheme were investigated, but only the octamethyl ester derivative was found, with no traces of the amide derivative. The results suggest that, in *Desulfovibrio*, the physiologically active prosthetic group may be an amidated form of siroheme.

Siroheme (**1**) is the prosthetic group of assimilatory sulfite and nitrite reductases found in many prokaryotes, and in the dissimilatory sulfite reductases of sulfate-reducing bacteria. The structure of the iron-free sirohydrochlorin (**2**) tetrapyrrole was deduced in a series of investigations (Murphy et al., 1973a; Murphy & Siegel, 1973; Battersby et al., 1977a,b; Scott et al., 1978) that also established its connection as an intermediate in the biosynthetic pathway leading to vitamin B_{12}. Although many of the key structural studies were performed on material isolated from cells of *Escherichia coli*, siroheme, often as the derivative sirohydrochlorin octamethyl ester (**3**), was detected in other organisms (Vega et al., 1975), especially *Desulfovibrio* strains (Murphy et al., 1973b; Battersby et al., 1977a), which have an active dissimilatory sulfur metabolism. While isolating the prosthetic group of sulfite reductases from various strains of *Desulfovibrio*, we discovered that the main recoverable product after demetallation and esterification was not sirohydrochlorin octamethyl ester (**3**), but a form with seven ester groups and one amide group stereospecifically at the 2^1-acetate (**4**). The purpose of this paper is to report the characterization of this new form that establishes its structure, and some data on the distribution of **4** among different cell lines.



1: M=Fe, R=H, X=OH
2: M=H,H, R=H, X=OH
3: M=H,H, R=CH_3, X=OCH_3
4:M=H,H, R=CH_3, X=NH_2
5: M=Fe, R=H, X=NH_2

MATERIALS AND METHODS

Desulfoviridin and the low-spin sulfite reductase (Lee et al., 1973a) and desulforubidin (Lee et al., 1973b) were purified according to previously published methods. Esterified tetrapyrroles were prepared from purified, lyophilized enzymes, or broken cell crude enzyme extracts that had also been lyophilized, mainly by the method of Scott et al. (1978), based upon anhydrous HCl in methanol, with exceptions that will be described when appropriate. We noted that ferrous salts could be omitted from the original procedure, since the strong acid seems sufficient to dissociate even ferric iron from the sirohydrochlorin core. As noted by previous workers, it is important to exclude oxygen during the esterification, to prevent the formation of mono- and bis-lactone derivatives of sirohydrochlorin (Battersby et al., 1977a,b). It was imperative to convert the prosthetic groups to the metal-free, ester derivatives for final purification. The carboxylic acid forms have strong detergent-like properties that make them extraordinarily difficult to purify for mass spectrometry and NMR at the available levels of these natural products. After workup, the dried, crude ester product mixture was purified by adsorption chromatography on silica gel. Crude ester products were applied to small silica columns equilibrated with neat chloroform and eluted with

† This work was supported in part by NIH Grants GM43292 (R.T.) and GM34903 (J.L.).

* Address correspondence to this author at the Department of Chemistry, University of Alabama, Tuscaloosa, AL 35487-0336. Phone: 205-348-8439. FAX: 205-348-9104. e-mail: rtimkovi@UA1VM.UA.EDU.

‡ University of Alabama.

§ University of Georgia.

⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1995.

0006-2960/95/0434-5248$09.00/0

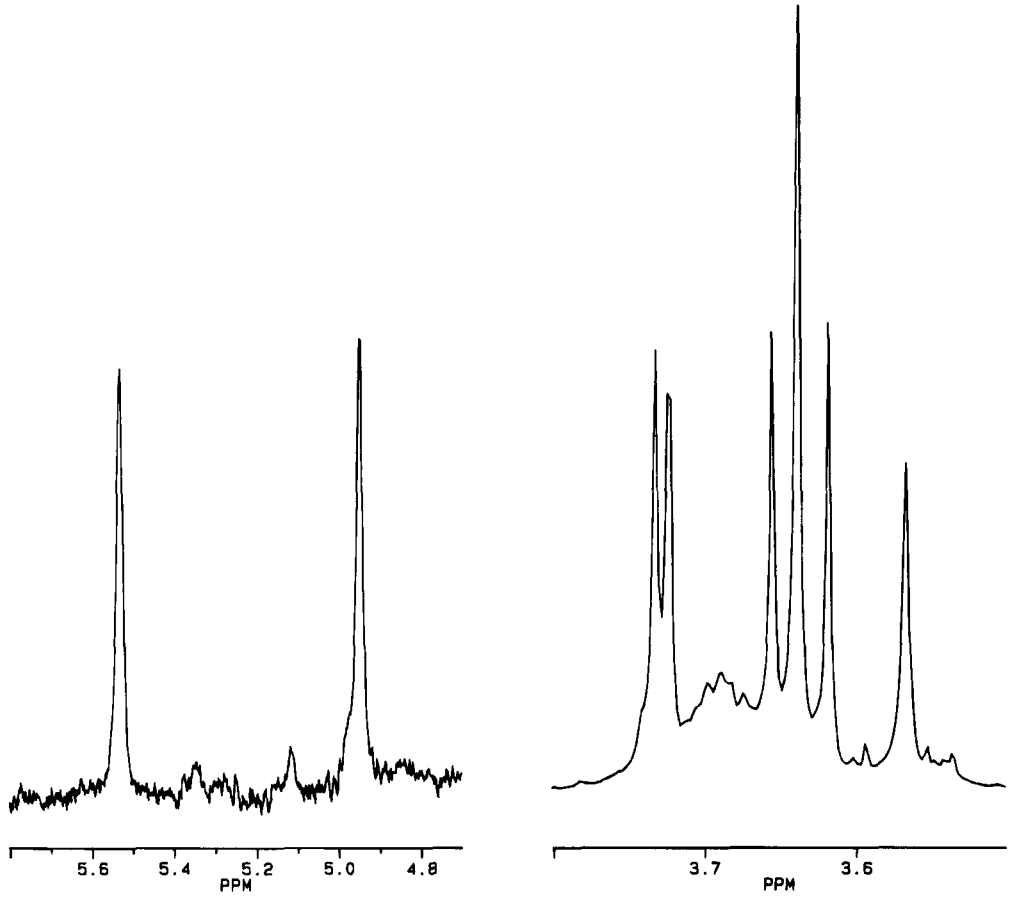


FIGURE 1: Portions of the ^{1}H-NMR spectrum of the isolated siroamide derivative that provide major evidence for the presence of an amide substituent. The left panel is from a spectrum taken in deuterated chloroform, that shows the two broad resonances assigned to the NH_2 moiety. The right panel is from a spectrum taken in deuterated methylene chloride that shows the presence of only seven methyl ester resonances, five resolved and two coincident. The two subspectra have different horizontal and vertical scales.

the same. The mobile fraction can be easily followed by its visible color or strong orange fluorescence. It was collected, dried, and further purified by HPLC (4.6 × 250 mm, 5 μm silica gel column, eluted with neat chloroform at 1 mL/min, detect at 400 nm, and the main sirohydrochlorin ester fraction elutes with capacity factor, k', of 2.9). If the initial low resolution column was subsequently eluted with neat ethyl acetate, a second and larger fraction of a sirohydrochlorin ester derivative was eluted. It was further purified by HPLC (eluted with neat ethyl acetate, k' in the range of 4−6 depending upon the moisture content of the solvent).

^{1}H-NMR spectra were recorded at 500 MHz at 295 K in either deuterated chloroform or deuterated methylene chloride, and the residual protic solvent peaks were used as chemical shift standards at 7.26 and 5.32 ppm, respectively. Liquid secondary ion mass spectrometry (LSIMS, also known as fast atom bombardment or FAB) was performed in a matrix that produces the $(M + H)^+$ ion as described previously (Musselmen et al., 1986).

RESULTS AND DISCUSSION

The structure of the first ester fraction was confirmed to be the authentic octamethyl ester **3** by NMR (Scott et al., 1978) and LSIMS (m/z of 975 for $(M + H)^+$). The more polar second ester derivative had a visible spectrum nearly indistinguishable from that of the octamethyl ester. Relative band intensities were the same, and band maxima in low resolution spectra appeared the same. In optical spectra taken under high resolution conditions with narrow slits (0.25 nm) recorded in the first or second derivative mode, the visible bands of the amide were oberved to be shifted to 1 nm lower wavelength than the octamethyl ester. Figure 3 of Murphy et al. (1973a) reports the octamethyl ester spectrum. Different chromatographic conditions have been used in the past by previous workers, but a general trend is that sirohydrochlorin octamethyl ester **3** behaves as a compound slightly less polar than uroporphyrin octamethyl ester. The first ester fraction described above was consistent with this behavior, but the second was far more polar than expected. From purified sulfite reductases, the relative yields were variable but on the order of 10:90 octamethyl ester to the second more polar ester derivative, while from whole cell extracts the amounts were 40:60. The isolation conditions promoted epimerization (Battersby et al., 1979) of both ester fractions, and the epimers were chromatographically resolved from the major isomers during HPLC (eluting before the major isomer) and constituted 30−40% of each.

Structure **4** was assigned to the polar ester on the following grounds. High resolution LSIMS indicated a molecular ion of 960.422 ± 0.002 (calcd 960.4242 for $C_{49}H_{62}N_5O_{15}$ as $(M + H)^+$). The matrix used has universally in our past experience produced the $(M + H)^+$ ion as the most intense LSIMS peak. This was confirmed in this case by running low resolution negative ion LSIMS that produces the $(M - 1)^-$ ion and observing an m/z of 958, and electron impact MS that gave a weak but observable 959 ion. The odd mass for the tetrapyrrole is unusual, but is consistent with the presence of an odd number of trivalent nitrogen atoms

Table 1: Proton Chemical Shifts and Assignments of the Isolated Sirohydrochlorin Octamethyl Ester and the Siroamide Heptamethyl Ester[a]

octamethyl ester			amide		
proton	shift	multiplcity, area	proton	shift	multiplicity, area
5	6.768	s, 1H	5	6.760	s, 1H
10	7.455	s, 1H	10	7.443	s, 1H
15	8.530	s, 1H	15	8.518	s, 1H
20	7.343	s, 1H	20	7.384	s, 1H
18^1, 12^1	4.282, 4.278	s, s, ~4H total	18^1	4.282	s
			12^1	4.280, 4.250	AB type $J = 15.8$ Hz, 4H total
13^1, 17^1	3.7−3.8	m	13^1, 17^1	3.7−3.8	m
13^2, 17^2	2.930	t, $J = 6.9$ Hz, 4H	13^2, 17^2	2.930, 2.914	t, t, $J = 6.9$ Hz
2^1, 7^1	2.745, 2.729		2^{1a}	2.910	d, $J = 13.8$ Hz, 5H total including 13^2, 17^2
	2.713	m[b], 4H	2^{1b}	2.672	d, $J = 13.8$ Hz, 1H
			7^1	2.707	s, ~2H
$3^{1'}$, $8^{1'}$	4.03−4.13	m, 2H total	$3^{1'}$	4.199	t, $J = 6.9$ Hz
			$8^{1'}$	4.028	dd, $J = 4.9$, 9.9 Hz
3^1, 8^1, 8^2	2.3−2.4	m	3^1, 8^{1b}, 8^2	2.3−2.4	m, ~5H
	2.0−2.1	m, ~6H total			
			8^{1a}	1.98	m, 1H
3^2	2.570	t, $J = 9$ Hz, ~2H	3^2	2.62, 2.48	m, m, ~2H total
$2^{1'}$, $7^{1'}$	1.818, 1.859	s, s, 3H each	$2^{1'}$	1.770	s, 3H
			$7^{1'}$	1.824	s, 3H
			NH_2	5.53, 4.95	br, 1H each
CH_3	3.713	s, ~3H	CH_3	3.713	s, ~3H
	3.707	s, ~3H		3.709	s, ~3H
	3.699	s, ~3H		3.697	s, ~3H
	3.658	s, ~6H		3.656	s, ~6H
	3.642	s, ~3H		3.636	s, ~3H
	3.601	s, ~3H		3.599	s, ~3H
	3.581	s, ~3H			

[a] The chemical shifts are in ppm nominally from tetramethylsilane, but residual chloroform was used as internal standard at 7.26 ppm at 295 K, recorded at 500 MHz. The reported shifts for the octamethyl ester are from material isolated in the present study and closely match an earlier report (Scott et al., 1978) taken under slightly different conditions. There are reproduced here to enable a close comparison to the amide derivative. Some resonances could only be integrated approximately because of overlap, while others were so obscured that no estimates were possible. [b] These resonances roughly appear as an apparent singlet at 2.745 and then a doublet at the other shift values, but are more likely two highly overlapped AB type subspectra.

(standard texts (Pavia et al., 1979) discuss the odd mass issue for common organic molecules). The NMR spectrum of **4** was extremely close to that of the octamethyl ester with two exceptions illustrated in Figure 1. Two broad one proton resonances were observed in the region 4.9−5.6 ppm in dry chloroform or methylene chloride. These disappeared upon addition of microliter amounts of 2% DCl in CD_3OD. The peaks were too broad to observe multiplicity, but homonuclear Hartmann−Hahn spectra indicated they were scalar coupled to each other, but nothing else. The methyl ester region of **4** in methylene chloride was fortuitously partially resolved at 500 MHz as shown and contained only six singlets, five of area corresponding to 3H and one of 6H. Complete NMR data for both ester derivatives are given in Table 1. Assignments followed from the previous report for sirohydrochlorin octamethyl ester and the ROESY spectra described next.

Evidence for the site of amidation was determined by nuclear Overhauser enhancements observed in ROESY spectra. Unfortunately, no NOE cross peaks were observed from the NH_2 protons, but this might not be surprising due to the broadness of these resonances and the fact that the carbonyl group may be rotating in solution. It was possible to assign the spin-coupled doublets at 2.9 and 2.7 ppm to the 2^1 methylene protons because of an NOE from the 20 meso proton to the 2.9 ppm doublet. Since these methylene resonances at 2.7 and 2.9 ppm are the most perturbed protons compared to the octamethyl ester, the amide was assigned to the 2^1 carbonyl. The 3^2 methylene protons are also perturbed in the amide compound compared to the octamethyl ester, but not as greatly as the 2^1 protons, presumably because the modification site is spatially close but with many intervening bonds.

We are extremely concerned over whether the isolated siroamide heptamethyl ester from *Desulfovibrio* is an artifact of the isolation procedure, or whether it reflects the presence of siroamide **5** as a functional prosthetic group. The strong acid conditions of esterification are not expected to promote amidation. No exogenous ammonia was added, and any traces present would be as ammonium ion. Although amides readily undergo acid hydrolysis, they can be resistant to acid-catalyzed methanolysis. The presence of some octamethyl ester with the majority as the monoamide, heptamethyl ester could reflect partial methanolysis of a sample originally with one amide and seven carboxylic acid groups. For tetrapyrrole carbonyls, this was tested by subjecting cyanocobalamin to our esterification and workup conditions. The unfractionated product mixture was analyzed by low resolution LSIMS. Solvolysis had clearly eliminated the nucleotide side chain, since the intense ions were around *m/z* of 960−1020. Multiple intense ions were observed, but in clusters corresponding to substitution of 1−2 amides by $-OCH_3$. Since there are seven amides in cobalamin (six NH_2 and one aminopropanol substitutent), roughly 80% of the possible amide bonds are resistant to methanolysis under these conditions.

It would be highly desirable to obtain direct evidence for or against an amidated heme within the intact sulfite reductases of *Desulfovibrio* without recourse to the isolation and derivatization manipulations, but this has not been

possible for us to do. As mentioned under Materials and Methods, we can only obtain spectroscopically pure material, from the starting levels available, after demetallation, esterification, and chromatography. As demonstrated by the final isolated material, the amide has negligible effect on the visible spectra, and so the visible spectra of the enzymes would not be expected to contain conclusive evidence. Resonance Raman studies have been performed on the enzyme (Lai et al., 1991), but the resultant spectra are complex and afford no clue for or against the presence of an amide (K. T. Yue, personal communication). The best evidence in hand is to consider the distribution of where siroamide has been discovered. It was the main form of sirohydrochlorin isolated in all the *Desulfovibrio* sources tested, including, *Desulfovibrio gigas* desulfoviridin and *D. gigas* whole cells, *Desulfovibrio vulgaris* desulfoviridin and the low-spin sulfite reductase, *Desulfovibrio desulfuricans* (Norway) desulforubidin, and *Desulfovibrio multispirans* desulfoviridin. Whole cells of *D. vulgaris* were extracted by the original method of Murphy et al. (1973a) and Murphy and Siegel (1973), and again more siroamide heptamethyl ester was isolated than the octamethyl ester, although in our hands this procedure also gave increased amounts of the monolactone. In investigations of tetrapyrroles produced by *Pseudomonas* (Harris et al., 1993), we isolated exclusively the octamethyl ester and detected no siroamide. A strain of *E. coli* overproducing the sulfite reductase (Wu et al., 1991) was kindly donated to us by Dr. N. M. Kredich. From whole cells we isolated only the octamethyl ester. The literature does contain reports of the octamethyl ester being isolated from *Desulfovibrio* sources (Murphy et al., 1973b; Battersby et al., 1977a). However, this was always detected in all of our *Desulfovibrio* isolations and was attributed to partial methanolysis of the amide. The previous workers may not have detected the amide because of its much stronger polarity, where it could be dismissed as incompletely esterified material, or because of a greater extent of methanolysis of the amide during esterification by the different conditions they used.

The significance of an amidated siroheme is only open to conjecture at this time. It is interesting to point out that sirohydrochlorin as precorrin-2 (Battersby, 1993) is an intermediate on the biosynthetic pathway to Factor F430, the nickel-containing tetrapyrrole prosthetic group (Pfaltz et al., 1982; Livingston et al., 1984) of the methylcoenzyme M reductase of methanogenic bacteria, and also on the pathway to vitamin B_{12}. F430 has an amidated acetate at 2^1 and a lactam at pyrrole ring B that could have involved an acetamide at 7^1 in its genesis. Vitamin B_{12} has exclusively amidated carboxylates, and recently the *cobB* gene and the resultant enzyme, described as a diamide synthase, have been characterized that lead to amidation at 2^1 and 7^1 (Crouzet et al., 1990). No corrinoid has been found so far in cells of *Desulfovibrio* species, but a protein containing a cobalt sirohydrochlorin (Battersby & Sheng, 1982) has been detected in both *D. gigas* (Moura et al., 1978) and *Desulfomicrobium baculatum* (Hatchikian, 1981) (formerly Desulfovibrio desulfuricans Norway 4 (Devereux et al., 1990)). It may be that, in *Desulfovibrio*, an amidation activity exists that is either a primitive precursor/ancestor of the F430 or B_{12} systems, or vestiges of a former system that is now disappearing through evolution. This does not necessarily mean that the amidation serves no function. It may be a premature, but highly intriguing, correlation that the sulfite reductases of *Desulfovibrio* are dissimilatory in nature, while the other sources are assimilatory.

SUPPLEMENTARY MATERIAL AVAILABLE

The NMR spectra of the isolated monoamide, heptamethyl ester derivative of sirohydrochlorin covering all assigned resonances in deuterated chloroform (Figure S1) and in deuterated methylene chloride (Figure S2) (2 pages). Ordering information is given on any current masthead page.

REFERENCES

Battersby, A. R. (1993) *Acc. Chem. Res. 26*, 15−21.

Battersby, A. R., & Sheng, Z. C. (1982) *J. Chem. Soc., Chem. Commun. 1982*, 1393−1394.

Battersby, A. R., Jones, K., McDonald, E., Robinson, J. A., & Morris, H. R. (1977a) *Tettrahedron Lett. 1977*, 2213−2216.

Battersby, A. R., McDonald, E., Morris, H. R., Thompson, M., Williams, D. C., Bykhovsky, V. Y., Zaitseva, N. I., & Bukin, V. N. (1977b) *Tetrahedron Lett. 1977*, 2217−2220.

Battersby, A. R., McDonald, E., Neier, R., & Thompson, M. (1979) *Chem. Commun. 1979*, 960−961.

Crouzet, J., Cauchois, L., Blanche, F., Debussche, L., Thibaut, D., Rouyez, M.-C., Rigault, S., Mayaux, J.-F., & Cameron, B. (1990) *J. Bacteriol. 172*, 5968−5979.

Devereux, D., He, S.-H., Doyle, C. L., Orkland, S., Stahl, D. A., Le Gall, J., & Whitman, W. B. (1990) *J. Bacteriol. 172*, 3609−3619.

Harris, W. F., III, Burkhalter, R. S., Lin, W., & Timkovich, R. (1993) *Bioorg. Chem. 21*, 209−220.

Hatchikian, E. C. (1981) *Biochem. Biophys. Res. Commun. 103*, 521−530.

Lai, K. K., Moura, I., Liu, M. Y., Le Gall, J., & Yue, K. T. (1991) *Biochim. Biophys. Acta 1060*, 25−27.

Lee, J.-P., Le Gall, J., & Peck, H. D., Jr. (1973a) *J. Bacteriol. 115*, 529−542.

Lee, J.-P., Yi, A., Le Gall, J., & Peck, H. D., Jr. (1973b) *J. Bacteriol. 115*, 453−455.

Livingston, D. A., Pfaltz, A., Schreiber, J., Eschenmoser, A., Ankel-Fuchs, D., Moll, J. Jaenchen, R., & Thauer, R. K. (1984) *Helv. Chim. Acta 67*, 334−351.

Moura, I., Moura, J. J. G., Santos, M. H., Xavier, A. V., & Le Gall, J. (1978) *FEBS Lett. 89*, 177−179.

Murphy, M. J., & Siegel, L. M. (1973) *J. Biol. Chem. 248*, 6911−6919.

Murphy, M. J., Siegel, L. M., Kamin, H., & Rosenthal, D. (1973a) *J. Chem. Biol. 248*, 2801−2814.

Murphy, M. J., Siegel, L. M., Kamin, H., DerVartanian, D. V., Lee, J.-P., Le Gall, J., & Peck, H. D., Jr. (1973b) *Biochem. Biophys. Res. Commun. 54*, 82−88.

Musselmen, B. D., Watson, J. T., & Chang, C. K. (1986) *Org. Mass Spectrom. 21*, 215−219.

Pavia, D. L., Lampman, G. M., & Kriz, G. S., Jr. (1979) *Introduction to Spectroscopy: A Guide for Students of Organic Chemistry*, pp 233−234, Saunders College Publishing Co., New York.

Pfaltz, A., Juan, B., Fässler, A., Eschenmoser, A., Jaenchen, R., Gilles, H. H., Diekert, G., & Thauer, R. K. (1982) *Helv. Chim. Acta 65*, 828−865.

Scott, A. I., & Santander, P. J. (1991) *in Biosynthesis of Tetrapyrroles* (Jordan, P. M., Ed.) pp 101−138, Elsevier, Amsterdam.

Scott, A. I., Irwin, A. J., Siegel, L. M., & Shoolery, J. N. (1978) *J. Am. Chem. Soc. 100*, 7987−7994.

Vega, J. M., Garrett, R. H., & Siegel, L. M. (1975) *J. Biol. Chem. 250*, 7980−7989.

Wu, J.-Y., Siegel, L. M., & Kredich, N. M. (1991) *J. Bacteriol. 173*, 325−333.

BI942637Z