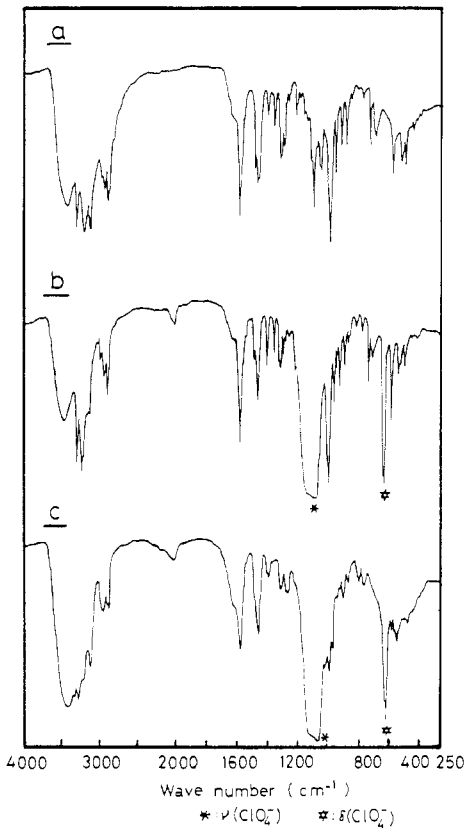


Figure 4. Infrared absorption spectra of (a) $Cr_2(taec)Cl_4$, (b) $Cu_2(taec)(ClO_4)_4$ (type 1), and (c) $Cu_2(taec)Br(ClO_4)_3$ (type 2).

of $Cr(en)_2I_2$, where en denotes ethylenediamine, has a corresponding band at 550 nm with a shoulder at 690 nm.[15] As shown in Figure 3, $Cr_2(taec)Cl_4$ obeys Curie's law and the effective magnetic moments are almost constant in the temperature range 85–300 K. The values of the effective magnetic moments, μ_{eff} = 4.70 μ_B (at 297 K) and 4.63 μ_B (at 85 K), were consistent with those of a chromium(II) ion of high spin state. The bromide also obeys Curie's law: μ_{eff} = 4.53 μ_B (at 297 K) and 4.53 μ_B (at 85 K). These results clearly indicate that magnetic interaction between the chromium(II) ions is very weak for both complexes.

As expected, the complexes are stable in dry air; no appreciable color change was observed for 1 month in dry air at ambient temperature. When exposed to moisture-saturated air, however, the compounds were tinged with brown in several minutes and became almost black in 2 h. A few air-stable binuclear chromium(II) complexes have been reported recently.[9-12] Cotton and his co-workers demonstrated that $Cr_2(hmp)_4$ (where Hhmp = 2-hydroxy-6-methylpyridine) was stable in dry air but was oxidized gradually in moist air after ca. 10 days.[9,10] Ardon et al. reported the compounds $[Cr_2(gly)_4X_2]X_2$ (where gly = zwitterionic glycine and X = Cl, Br), which were quite stable toward atmospheric oxidation in the solid state.[11] The mononuclear chromium(II) bis(nicotinate) complex *trans*-$[Cr(nic\text{-}N)_2(H_2O)_4]$ (where nic-N^- is N-coordinated nicotinate ion), reported by Broderick et al. was also stable in air. The remarkable stability of $[Cr_2(gly)_4X_2]X_2$ and *trans*-$[Cr(nic\text{-}N)_2(H_2O)_4]$ was attributed to the tight hydrogen bondings within the crystal lattice.[11,12] The binuclear complexes $Cr_2(hmp)_4$ and $[Cr_2(gly)_4X_2]X_2$ have a quadruple Cr–Cr bond, which should substantially contribute to the stability. However, in the case of our complexes, neither direct Cr–Cr bonding nor very strong hydrogen bondings like those of the above complexes are likely to exist. Hydrogen bondings much stronger than those in $Cr(en)_2I_2$, which readily undergoes air oxidation, cannot be expected for our complexes. Thus, the stability of our complexes toward air may be attributed to the steric effect of the taec ligand as observed for cobalt(II) taec complexes.

All attempts to obtain single crystals for X-ray diffraction have been unsuccessful so far. In order to elucidate the structure of the complexes, their infrared spectra were compared with those of copper(II) taec complexes whose structures were already known. It was shown in a preceding paper[4] that the type of structure of taec complexes could be diagnosed by comparing IR spectra. As shown in Figure 4, the IR spectral pattern of $Cr_2(taec)Cl_4$ is very similar to that of $Cu_2(taec)(ClO_4)_4$ (type 1), except for the absorption due to ClO_4^-, but is substantially different from that of $Cu_2(taec)Br(ClO_4)_3$ (type 2). The spectrum of $Cr_2(taec)Br_4$ was practically the same as that of the chloride. These results indicate that the present complexes have the type 1 structure. If so, the methylene groups on the 6- and 13-positions of the cyclam ring are located near the coordination sites of the metal centers, hindering the formation of six-coordination. In the case of $Cr_2(hmp)_4$,[9,10] the axial coordination sites are blocked by the methyl groups of the ligands. Hence, the high stability of this complex in air may partly be due to the steric configuration as observed for our complexes.

Acknowledgment. We wish to thank Prof. H. Ōkawa of Kyushu University for the magnetic susceptibility measurements and Prof. M. Mikuruya of Kwansei Gakuin University for his helpful advice and discussions. This work was partly supported by a Grant-in-Aid for Scientific Research (No. 62540478) from the Ministry of Education, Science and Culture.

(15) Earnshow, A.; Larkworthy, L. F.; Patal, K. C. *J. Chem. Soc. A* **1969**, 1339.

Contribution from the Institute of Chemistry, College of General Education, Osaka University, Toyonaka, Osaka 560, Japan, Laboratory of Chemistry, College of Liberal Arts and Sciences, Kanazawa University, Kanazawa 920, Japan, Department of Environmental Science, Faculty of Integrated Arts and Sciences, Hiroshima University, Naka-ku, Hiroshima 730, Japan, and Department of Foreign Languages, Nagoya University of Foreign Studies, Nisshincho, Aichigun, Aichi 470-01, Japan

Spectroscopic Characterization of Cobalt(II)-Substituted *Achromobacter* Pseudoazurin: Similarity of the Metal Center in Co(II)-Pseudoazurin to Those in Co(II)-Plastocyanin and Co(II)-Plantacyanin

Shinnichiro Suzuki,*[1a] Takeshi Sakurai,[1b] Sohsuke Shidara,[1c] and Hidekazu Iwasaki[1d]

Received April 27, 1988

Pseudoazurin is a bacterial blue copper protein, together with azurin, amicyanin, and rusticyanin.[2] There are three pseudoazurins so far isolated. Two proteins from denitrifying bacteria, *Achromobacter cycloclastes* IAM 1013[3,4] and *Alcaligenes faecalis* strain S-6[5] have one blue copper per 124[6] and 123[7] amino acid residues, respectively, being able to transfer electrons to their

(1) (a) Osaka University. (b) Kanazawa University. (c) Hiroshima University. (d) Nagoya University of Foreign Studies.
(2) Adman, E. T. In *Metalloproteins, Part 1: Topics in Molecular and Structural Biology*; Harrison, P. M., Ed.; MacMillan Press: London, 1985; pp 1–42.
(3) Iwasaki, H.; Matsubara, T. *J. Biochem.* **1973**, *73*, 659–661.
(4) Liu, M.-Y.; Liu, M.-C.; Payne, W. J.; Legall, J. *J. Bacteriol.* **1986**, *166*, 604–608.
(5) Kakutani, T.; Watanabe, H.; Arima, K.; Beppu, T. *J. Biochem.* **1981**, *89*, 463–472.
(6) Ambler, R. P. In *The Evolution of Metalloenzymes, Metalloproteins and Related Materials*; Leigh, G. J., Ed.; Symposium Press: London, 1977; pp 100–118.
(7) Hormel, S.; Adman, E. T.; Walsh, K. A.; Beppu, T.; Titani, K. *FEBS Lett.* **1986**, *197*, 301–304.

0020-1669/89/1328-0802$01.50/0

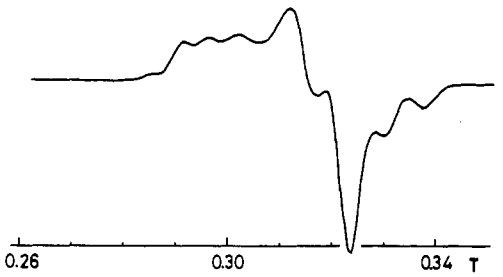


Figure 1. EPR spectrum of *Achromobacter* pseudoazurin in 0.1 M phosphate buffer (pH 6.0) at 77 K.

homologous copper nitrite reductases.[4,5] The other pseudoazurin synthesized along with amicyanin by methylotroph (*Pseudomonas* AM1) growing on methylamine contains 123 amino acid residues.[8,9] The visible absorption spectrum of *Achromobacter* pseudoazurin displays three absorption bands at 455 nm (ϵ = 1400 M^{-1} cm^{-1}), 596 nm (ϵ = 3700 M^{-1} cm^{-1}), and 754 nm (ϵ = 1700 M^{-1} cm^{-1}) like those of the other bacterial copper proteins, but the ϵ value of the 455-nm band is characteristically larger than those (300–600 M^{-1} cm^{-1}) of the corresponding bands of azurin[10,11] and amicyanin:[12,13] in the case of *Alcaligenes* pseudoazurin, the ϵ value is 1200 M^{-1} cm^{-1}.[5] The 450-nm absorption band of rusticyanin also has a large absorption coefficient (1060 M^{-1} cm^{-1}).[14]

Figure 1 shows the EPR spectrum of *Achromobacter* pseudoazurin at 77 K. The rhombic character (g_z = 2.20, g_y = 2.09, g_x = 2.02, A_z = 55 G, and A_x = 68 G) of the copper(II) center, identical with that in *Alcaligenes* pseudoazurin,[5] is clearly observed, whereas axial-type spectra ($g_\parallel > g_\perp > 2$) consistent with a $d_{x^2-y^2}$ ground state are recognized in azurin[2,10,11,15] and amicyanin,[16] which exhibit a weak absorption band at around 450 nm. The similar rhombic EPR signal was also observed in rusticyanin.[17] Moreover, it is interesting that the different symmetries of EPR signals were found in simple blue copper proteins from plants (rhombic in plantacyanin,[18,19] stellacyanin,[20] and mavicyanin;[21] axial in plastocyanin[2,22,23] and umecyanin[24]).

Recently the three-dimensional structure of *Alcaligenes* pseudoazurin was determined at 2.9-Å resolution by X-ray crystallography.[25] The crystal analysis demonstrated that the structure

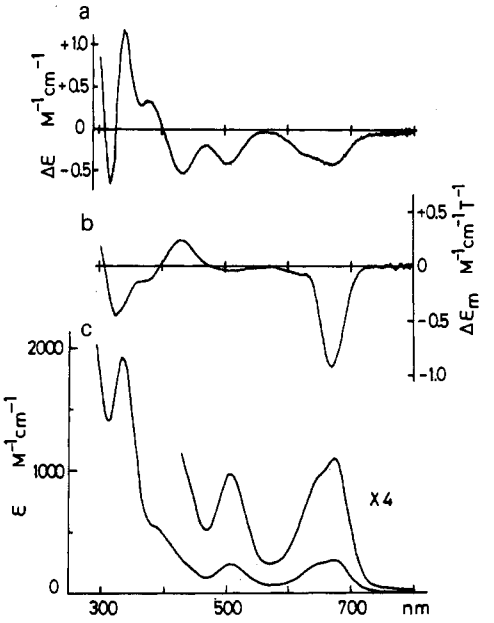


Figure 2. CD (a), MCD (b), and absorption (c) spectra of Co(II)-substituted *Achromobacter* pseudoazurin in 0.1 M Tris–HCl (pH 8.0) at room temperature.

Table I. Absorption Spectral Data for Cobalt(II) Derivatives of Blue Copper Proteins (λ, nm)

	A. cycloclastes pseudoazurin	cucumber plantacyanin	cucumber plastocyanin	bean plastocyanin
$S^- \rightarrow Co$	335	331	336	333
	390 (sh)[a]	390 (sh)	380 (sh)	385 (sh)
	440 (sh)	440 (sh)	430 (sh)	430 (sh)
d → d	505	510	507	508
	640 (sh)	640 (sh)	650	650 (sh)
	673	676	676	673
ref	this work	34	37	32

	Alcaligenes sp. azurin	*P. aeruginosa* azurin	stellacyanin
$S^- \rightarrow Co$	329	330	310
	374	375	365
	410 (sh)	408 (sh)	
d → d	522	521	540
	638	637	625
	655 (sh)		655
ref	unpublished data	32	31,32

[a] sh = shoulder band.

of an eight-stranded β-barrel resembles closely those of plastocyanin[26,27] and azurin,[11,28–30] and the copper center also has a donor set containing two histidine residues, one cysteine, and one methionine like those of plastocyanin and azurin. The geometry of the copper site, however, is still not disclosed.

Here we report the preparation and characterization of Co(II)-substituted *Achromobacter* pseudoazurin in order to obtain the structural information of the copper center. Copper ions in many blue copper proteins have been replaced by cobalt(II) ions generally suitable as an environmental probe.[31–36]

(8) Tobari, J. in *Microbial Growth on C_1 Compounds*; Crawford, R. L., Hanson, R. S., Eds.; American Society for Microbiology: Washington, DC, 1984; pp 106–112.
(9) Ambler, R. P.; Tobari, J. *Biochem. J.* **1985**, *232*, 451–457.
(10) Brill, A. S.; Bryce, G. F.; Maria, H. J. *Biochim. Biophys. Acta* **1968**, *154*, 342–351.
(11) Ainscough, E. W.; Bingham, A. G.; Brodie, A. M.; Ellis, W. R.; Gray, H. B.; Loehr, T. M.; Plowman, J. E.; Norris, G. E.; Baker, E. N. *Biochemistry* **1987**, *26*, 71–82.
(12) Tobari, J.; Harada, Y. *Biochem. Biophys. Res. Commun.* **1981**, *101*, 502–508.
(13) Husain, M.; Davidson, V. L.; Smith, A. J. *Biochemistry* **1986**, *25*, 2431–2436.
(14) Cox, J. C.; Boxer, D. H. *Biochem. J.* **1978**, *174*, 497–502.
(15) Ryden, L. In *Copper Proteins and Copper Enzymes*; Lontie, R., Ed.; CRC Press, Inc.: Boca Raton, FL, 1984; Vol. 1, pp 158–182.
(16) Husain, M.; Davidson, V. L. *J. Biol. Chem.* **1985**, *260*, 14626–14629.
(17) Cox, J. C.; Aasa, R.; Malmström, B. G. *FEBS Lett.* **1978**, *93*, 157–160.
(18) Aikazyan, V. T.; Nalbandyan, R. M. *Biochim. Biophys. Acta* **1981**, *667*, 421–432.
(19) Aikazyan, V. T.; Nalbandyan, R. M. *FEBS Lett.* **1975**, *55*, 272–274.
(20) Malmström, B. G.; Reinhammar, B.; Vanngard, T. *Biochim. Biophys. Acta* **1970**, *205*, 48–57.
(21) Marchesini, A.; Minelli, M.; Merkle, H.; Kroneck, P. M. H. *Eur. J. Biochem.* **1979**, *101*, 77–84.
(22) Lappin, A. G. In *Copper Proteins*; Sigel, H., Ed.; Metal Ions in Biological Systems, Vol. 13; Marcel Dekker, Inc.: New York, 1981: p 15–71.
(23) Suzuki, S.; Sakurai, T.; Nakajima, T. *Plant Cell Physiol.* **1987**, *28*, 825–831.
(24) Stigbrand, T.; Malmström, B. G.; Vanngard, T. *FEBS Lett.* **1971**, *12*, 260–262.
(25) Petratos, K.; Banner, D. W.; Beppu, T.; Wilson, K. S.; Tsernoglou, D. *FEBS Lett.* **1987**, *218*, 209–214.
(26) Guss, J. M.; Freeman, H. C. *J. Mol. Biol.* **1983**, *169*, 521–563.
(27) Guss, J. M.; Harrowell, P. R.; Murata, M.; Norris, V. A.; Freeman, H. C. *J. Mol. Biol.* **1986**, *192*, 361–387.
(28) Adman, E. T.; Stenkamp, R. E.; Sieker, L. C.; Jensen, L. H. *J. Mol. Biol.* **1978**, *123*, 35–47.
(29) Norris, G. E.; Anderson, B. F.; Baker, E. N. *J. Mol. Biol.* **1983**, *165*, 501–521.
(30) Norris, G. E.; Anderson, B. F.; Baker, E. N. *J. Am. Chem. Soc.* **1986**, *108*, 2784–2785.
(31) McMillin, D. R.; Holwerda, R. A.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 1339–1341.
(32) McMillin, D. R.; Rosenberg, R. C.; Gray, H. B. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 4760–4762.

The pseudoazurin isolated from *A. cycloclastes* IAM 1013 was purified according to the methods reported already.[3,4] The absorbance ratio of A_{282}/A_{594} was 1.45. The complete Cu depletion was carried out by dialyses (twice) of the pseudoazurin solution against 0.1 M Tris-HCl buffer (pH 8.0) containing KCN (0.01 M). Cobalt(II)-substituted pseudoazurin was prepared by anaerobic incubation of apopseudoazurin (0.49 μmol) and $CoCl_2$ (5 μmol; Co in 99.999% purity) in 2 mL of 0.1 M Tris-HCl buffer (pH 8.0) for 6 days at 4 °C. The excess cobalt ion was removed by dialysis against 0.1 M Tris-HCl (pH 8.0) for 5 h and the resulting Co(II)-pseudoazurin was concentrated for spectroscopic measurement. The concentration of cobalt in Co(II)-pseudoazurin was determined to be 0.8 mM (Co/protein = 1) by measurement with an atomic absorption spectrophotometer.

The electronic, CD, and MCD spectra of Co(II)-pseudoazurin at room temperature are represented in Figure 2. The electronic absorption spectrum in the range of 300-500 nm shows one absorption band at 335 nm and two shoulder bands at around 390 and 440 nm. In the same region, the CD and MCD spectra manifest three and four extrema, respectively. These absorption, CD, and MCD bands demonstrated that there is one cysteinyl ligand around cobalt(II) responsible for a $S^- \rightarrow$ Co(II) charge transfer, as described in the other Co(II)-substituted blue copper proteins (Table I). This finding corresponds with the results of the amino acid sequence and the X-ray crystallographic analysis of *Alcaligenes* pseudoazurin.[25] The visible absorption peaks at 505 and 673 nm and the shoulder band near 640 nm (ϵ = 240-270 M^{-1} cm^{-1} are assigned to the d-d transitions ($^4A_4(F) \rightarrow {}^4T_1(P)$) for the distorted tetrahedral Co(II) ion,[33,38] which are compared with the d-d transitions for several Co(II)-substituted proteins in Table I. The negative pattern of the MCD spectrum at 600-700 nm is likewise compatible with tetrahedral symmetry for the high-spin Co(II) ion.[33,38] In Table I, the spectral data of Co(II)-substituted *Achromobacter* pseudoazurin are similar to those of the Co(II) derivatives of two plastocyanins and especially cucumber plantacyanin, but they are a little different from those of the Co(II) derivatives of two azurins and stellacyanin. Moreover, the CD and MCD spectra of Co(II)-pseudoazurin in Figure 2 also display more of a resemblance to those of Co(II)-plastocyanin and Co(II)-plantacyanin than to those of Co(II)-azurin and Co(II)-stellacyanin.[33,34,37]

The Co(II) substitution of pseudoazurin implies that the donor set of the copper site in native pseudoazurin is in accord with that of cucumber plantacyanin and the structure of the copper site is essentially more analogous to those in cucumber and bean plastocyanins rather than that in bacterial azurin. According to the X-ray crystallographic analyses of plastocyanin[26,27] and azurin,[11,28-30] the copper site of plastocyanin was determined to be a very distorted tetrahedral arrangement with three in-plane, strongly bound ligands (two imidazole nitrogens and one cysteinyl sulfur, CuN_2S) and one axial, weakly bound sulfur ligand, methionine.[26] However, in azurin, a distorted trigonal-planar geometry (CuN_2S) with two weakly interacting groups (methionyl sulfur and the peptide carbonyl oxygen of a glycine residue) in axial positions completing an axially elongated trigonal bipyramid was confirmed.[11,30] The spectroscopic difference between Co(II)-plastocyanin and Co(II)-azurin could be associated with the above geometrical differences in the native forms.

As mentioned above, the intense absorption bands near 450 nm and the rhombic-type EPR signals of native pseudoazurin and plantacyanin[39] are distinct from the weak corresponding absorption band and the axial-type EPR signal of native plastocyanin. On the other hand, the metal sites in the Co(II) derivatives of pseudoazurin and plantacyanin are geometrically similar to that in Co(II)-plastocyanin. This suggests that the axial binding of methionine sulfur to the distorted trigonal-planar CuN_2S moiety in pseudoazurin or plantacyanin may be stronger than that in plastocyanin. It seems likely that the axial bonds in the copper sites of pseudoazurin and plantacyanin, which are shorter than that in plastocyanin, cause a rhombic EPR signal attributable to a distorted tetrahedral copper(II) and contribute to the high absorptivity of the band near 450 nm. The 455-nm band of *Achromobacter* pseudoazurin consists of two transitions exhibiting two CD extrema at 400 and 464 nm; at least one of the two transitions is probably due to a S(Met) $\rightarrow$ Cu charge-transfer transition,[36,40] although an alternative assignment to N(His) $\rightarrow$ Cu charge transfers is considered a possibility.[40,41] Native stellacyanin also shows a rhombic EPR spectrum. However, the spectroscopic properties of Co(II)-stellacyanin are different from those of Cu(II)-pseudoazurin, which is likely related to the absence of methionine in the coordination sphere of stellacyanin.[2]

Registry No. Cys, 52-90-4; Co, 7440-48-4; Cu, 7440-50-8.

(33) Solomon, E. I.; Rawlings, J.; McMillin, D. R.; Stephens, P. J.; Gray, H. B. *J. Am. Chem. Soc.* **1976**, *98*, 8046-8048.
(34) Sakurai, T. *Biochem. Biophys. Res. Commun.* **1986**, *139*, 961-966.
(35) Suzuki, S.; Sakurai, T.; Nakahara A.; Masuko, M.; Iwasaki, H. *Biochim. Biophys. Acta* **1985**, *827*, 190-192.
(36) Tennent, D. L.; McMillin, D. R. *J. Am. Chem. Soc.* **1979**, *101*, 2307-2311.
(37) Suzuki, S.; Yasui, S.; Kawahara, K.; Sakurai, T.; Okamoto, H.; Sawada, S.; Hirose, S.; Nakahara, A.; Yang, W. *Sci. Rep., Coll. Gen. Educ. Osaka Univ.* **1985**, *34*, 1-6.
(38) Vallee, B. L.; Holmquist, B. In *Methods for Determining Metal Ion Environments in Proteins*; Darnall, D. W., Wilkins, R. G., Eds.; Advances in Inorganic Biochemistry, Vol. 2; Elsevier: New York; p 27-74.
(39) In the absorption spectrum of cucumber plantacyanin, the value at 443 nm was reported to the 2030 M^{-1} cm^{-1}: Murata, M.; Begg, G. S.; Lambrou, F.; Leslie, B.; Simpson, R. J.; Freeman, H. C.; Morgan, F. J. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 6434-6437.
(40) Penfield, K. W.; Gay, R. R.; Himmelwright, R. S.; Eickman, N. C.; Norris, V. A.; Freeman, H. C.; Solomon, E. I. *J. Am. Chem. Soc.* **1981**, *103*, 4382-4388.
(41) Fawcett, T. G.; Bernarducci, E. E.; Krogh-Jespersen, K.; Schugar, H. J. *J. Am. Chem. Soc.* **1980**, *102*, 2598-2604.

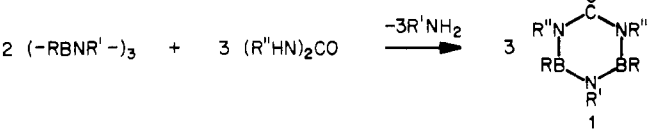
Contribution from the Department of Chemistry, University of Kentucky, Lexington, Kentucky 40506-0055

Reactions of *N*,*N*'-Dimethylurea with Boron-Nitrogen Compounds[1]

L. Komorowski† and K. Niedenzu*

Received August 30, 1988

Only two studies on the interaction of boron-nitrogen compounds with ureas are known. First, the reaction of borazines, $(-RBNR'-)_3$, with urea as well as *N*-mono- and *N*,*N*'-diorganyl derivatives thereof has been described to proceed according to eq 1. The resultant 1,3,5-triaza-2,4-diboracyclohexan-6-one



1 (1)

(=ketotriazadiborinane) species **1** (obtained in 55-65% yield) were characterized by elemental analysis only.[2] Several compounds containing the same structural skeleton of **1** and related species such as **2** and **3** were found among the reaction products of haloorganylboranes with N-organo-substituted ureas as well as N-lithiated[3] or N-silylated[4,5] derivatives of the latter.

†On leave of absence from the Technical University of Wrocław, Wrocław, Poland.

0020-1669/89/1328-0804$01.50/0