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SYNTHESIS OF A FLUORESCENT BORONIC ACID WHICH REVERSIBLY BINDS TO CELL WALLS AND A DIBORONIC ACID WHICH AGGLUTINATES ERYTHROCYTES

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Summary

N-(5-dimethylamino-1-napthalene sulfonyl)-3-aminobenzene boronic acid (Dns-PBA) and N,N'-bis-3(dihydroxylborylbenzene)adipamide (Bis-PBA) were synthesized. The former is found to reversibly associate with Bacillus subtilis, apparently through boronate diester linkages with carbohydrates on the cell surface. The latter displays the lectin-like property of agglutinating red blood cells.

INTRODUCTION

Carbohydrate complexes with borate anion in aqueous solution have been known for a long time [1,2], and corresponding complexes with boronate anion, $[R\text{-}B(OH_3)]^-$, have been described [3]. A recent review of chemical studies on boronic acids and their carbohydrate derivatives [4] indicated that most studies have been done in non-aqueous solvents. The pioneering work of Gilham and his colleagues [5] with boronic acids attached to insoluble supports, however, clearly showed that these derivatives were useful for separating sugars, nucleosides and nucleotides in aqueous solution. In addition boronate-containing column supports have been used for: (a) the isolation of 3'-terminal polyribonucleotides of bacteriophages Q_β and f2 [6], (b) the isolation of proteins containing ADP-ribosyl groups [7], (c) the isolation of tRNA species [8-10], (d) the isolation of catecholamines from urine [11], (e) the detection of modified nucleosides [12,13], (f) the assay of ribonucleotide reductase [14], (g) the assay of adenylyl cyclase [15], (h) the assay of c-AMP phosphodiesterase [16], and (i) the group specific affinity chromatography of UDP-glucose pyrophosphorylase [17]. We report here the synthesis of a fluorescent boronic acid which reversibly associated with Bacillus subtilis, presumably through diester linkages at the cell surface, and a diboronic acid which agglutinated red blood

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cells. (A portion of this work was presented at the XIth International Congress of Biochemistry, Toronto, Canada, July 8-13, 1979, in abstract 07-5-H91).

MATERIALS AND METHODS

A fluorescent boronic acid, N-(5-dimethylamino-1-naphthalene sulfonyl)-3-aminobenzene boronic acid (Dns-PBA), was synthesized by mixing 3.0 mmoles of m-aminobenzeneboronic acid hemisulfate (Sigma) in 70 ml of 0.75 M phosphate buffer, pH 6.5, with 3.7 mmoles of dansyl chloride (Sigma) in 90 ml of acetone at 30°C. After 2 hours, 30 mmoles of mannitol were added, and the pH was adjusted to 9.9 with 1 M NaOH. Acetone was removed by means of a rotatory evaporator, and the clear aqueous solution was extracted with four 30 ml portions of diethyl ether. The aqueous layer was acidified with 1 M HCl to pH 4.1, and the cloudy solution extracted with four 30 ml portions of diethyl ether; these ether extracts were then pooled and washed with four 20 ml portions of deionized water and the ether removed in a rotatory evaporator to yield an amorphous green powder. Thin layer chromatography on Bakerflex IBF silica gel plates in 50% aqueous acetone showed a single, fluorescent spot. 100 MHz ^{1}H NMR (CD_3SOCD_3, TMS): δ 2.79 (s, 6, CH_3), 7.1-8.5 (m, 12, Ar-H + OH), 10.50 (s, 1, SO_2NH). Elemental analysis (Galbraith Laboratories, Knoxville, Tenn.) of the hydrochloride salt (prepared by dissolving the above powder in 0.1 M HCl followed by lyophilization) was consistent with the hydrochloride trihydrate salt of the proposed compound. Anal.: C, 47.08%; H, 5.27%; B, 2.37%; N, 5.90%; S, 6.88%.

Cells from a single colony on a gelatin plate were grown in Hanson's supplemented nutrient broth [18] (Difco), harvested 3 hours after the end of logrithmic growth by centrifugation at 10,000 x g for 5 min, and stored frozen. All solutions contained 50 mM HEPPS buffer (Sigma Chemical Co.) and were at pH 8.5; centrifugation after each step was at 730 x g for 10 min. Cell samples (0.16 g dry weight) were washed with 3 ml aliquots of buffer, centrifuged, then resuspended in either 3 ml of buffer or 3 ml of buffer containing 10 mM mannitol for 15 min. at room temperature, centrifuged, and the supernatant solution discarded. To each sample of cells was added 2 ml of 0.35 mM dansylated benzene boronic acid; each tube was incubated 15 min. at room temperature, centrifuged and the supernatant solutions discarded. Finally, cells were washed with 3 ml aliquots of buffer or mannitol-buffer mix, such that cells pre-incubated with mannitol were the ones washed with mannitol. Fluorescence of these washes was read on a Turner 110 Fluorometer using #110-811 filter (color spec. 7-60) for excitation and #110-817 (color spec. 8) for emission. Treatments were in triplicate.

Guar beads were made from a high molecular weight galacto-mannosyl polymer (Stein-Hall Specialty Chemicals) by cross-linking with epichlorohydrin by the procedure of Determann [19]. Guar bead samples (0.05 g dry weight) were treated similarly to the cells except the mannitol preincubation was omitted and treatments were in duplicate.

To 5 mmoles of m-aminobenzeneboronic acid hemisulfate (Sigma) in 30 ml of water adjusted to pH 4.7 with NaOH and cooled to 9°C was added 5 mmoles of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (Sigma). To this stirred solution was added, at a rate of 48 ml/hr, 30 ml of an aqueous solution of 2.5 mmole of adipic acid (Sigma) at pH 4.7; temperature was maintained at 9°C. The mixture was stirred for 1 hr. after addition was complete and stored at 4°C overnight. The white precipitate was collected by filtration and washed with water, dissolved in methanol, and crystallized from water to obtain white needles. Yield: 50.4%. Melting point: discoloration at 214°C, melting complete at 217°C. 100 MHz ^{1}H NMR (CD_3SOCD_3, TMS): δ 1.70 (m, 2, CH_2), 2.38 (m, 2, CH_2), 7.20-7.75 (m, 4, Ar-H), 7.92 (s, 2, OH), 9.72 (s, 1, CON-H). The infrared spectrum was also consistent with proposed structure. Elemental analysis (Galbraith Laboratories, Knoxville, Tenn.) was consistent with the proposed structure. Anal.: C, 56.19%; H, 6.04%; B, 5.81%; N, 7.09%; and O, 24.87%.

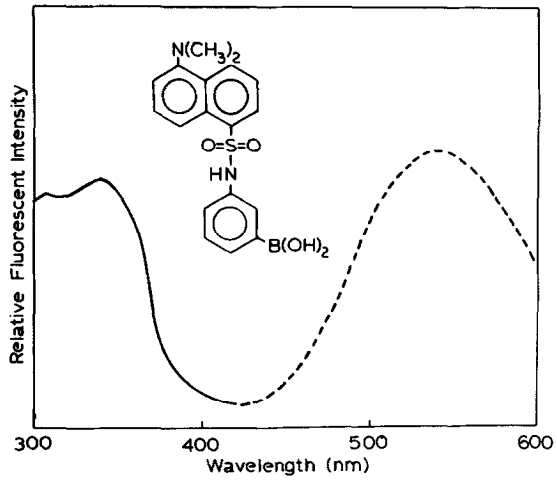

Figure 1. Structure and fluorescence spectrum of N-(5-dimethylamino-1-napthalene sulfonyl)-3-aminobenzene boronic acid (Dns-PBA). Emission (---) and excitation (——) spectra of a 0.9 mM solution of this compound in 0.05 M HEPPS buffer, pH 8.5, were taken on an Aminco-Bowman spectrofluorometer, using fixed wavelengths of 340 nm and 550 nm, respectively.

RESULTS AND DISCUSSION

The observation that m-aminobenzeneboronic acid caused selective inhibition of sporulation in Bacillus subtilis [20] suggested that this compound might be acting through esterification to diols at the cell surface. To test this idea, the fluorescent derivative shown in Figure 1 was synthesized.

Initial qualitative studies with this fluorescent boronic acid showed that cells of Bacillus subtilis became visibly fluorescent when exposed to Dns-PBA and that the fluorescence could be partially displaced by benzeneboronic acid and nearly completely displaced by mannitol. The more quantitative experiment of Table 1 confirms that B. subtilis can bind Dns-PBA, a fraction of which can

TABLE 1. Binding of Dns-PBA to Bacillus subtilis and to guar beads.

	nmoles of dansylated boronic acid in wash	
Wash buffer	B. subtilis cells	Guar beads
HEPPS alone	125±2	27±1
HEPPS + mannitol	271±13	64±3
Ratio[a]	0.46	0.42

[a]Fluorescence in HEPPS wash/fluorescence in HEPPS-mannitol wash.

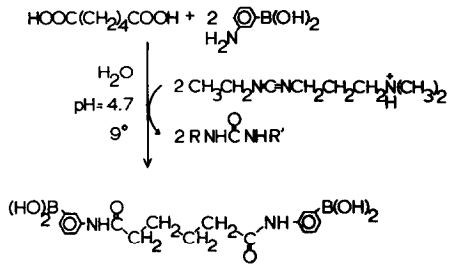

Figure 2. Synthesis and structure of N,N'-bis-3(dihydroxyborylbenzene) adipamide.

be displaced by mannitol. Preliminary studies indicate that sporulating cells bind 2- to 3-fold more boronate per mg of cell (dry wt.) than vegetative cells.

The guar beads (Table 1), which should have many exposed cis-diols, also bound Dns-PBA; approximately the same fraction of fluorescence was displaced by mannitol from the beads as from the cells. We suggest that at least that fraction of boronate which can be displaced from B. subtilis by mannitol is binding to the cell surface by esterification to cis-diols.

Recently, Gallop and Paz have reported that Dns-PBA is rapidly taken up by lysozomes and may be useful as a vital stain [21].

The ability of the boronate to bind to a bacterial cell surface, presumably through carbohydrate residues, suggested that a double-headed boronate derivative might exhibit agglutinating properties, in a sense act as an artificial lectin. The synthesis of several types of diboronic acids has been described [22]; the synthesis of a new double-headed boronic acid, Bis-PBA, is shown in Figure 2.

As shown in Figure 3, this double-headed reagent, like lectins, can cause the agglutination of erythrocytes. Since the cells in the control tubes, which contained twice the molar concentration of monofunctional boronic acid, were not agglutinated but formed the usual "buttons" in the bottom of the tubes, it seems likely that the agglutinated cells were cross-linked by the double-headed reagent. Similar agglutination patterns were observed when type O human erythrocytes or sheep erythrocytes were used. Upon standing at room temperature for several months, the solid diboronic acid undergoes decomposition, probably deboronation [25], as several spots were observed when this material was chroma-

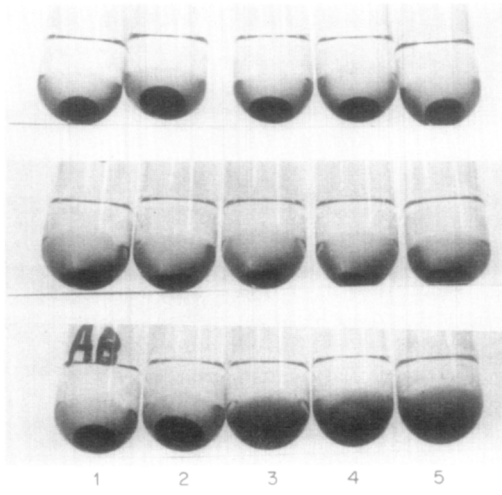

Figure 3. Agglutination of Human Erythrocytes with Bis-PBA. Fresh human AB erythrocytes were washed and stored in Alsever's solution [23] at 4°C. Immediately before use, a 5% suspension of erythrocytes was made in 0.2 M HEPES buffer (Sigma Chem. Co.,), pH 8.5, which was 0.9% in NaCl [24]. The Bis-PBA and benzeneboronic acid were dissolved in 2-ethoxy ethanol. All tubes contained a 0.25% suspension of erythrocytes and 0.18 mmoles of HEPES at a final pH of 8.5 in a total volume of 1.0 ml. In addition, tubes contained the following final concentrations. All rows contained 2-ethoxy ethanol; by column: 1, 1.5%; 2, 2.5%; 3, 4%, 4, 5%; 5, 10%. The middle row of tubes contained increasing amounts of benzeneboronic acid; by column: 1, 0.6 mM; 2, 1.0 mM; 3, 1.6 mM; 4, 2.5 mM; 5, 4.0 mM. The bottom row of tubes contained increasing amounts of the Bis-PBA; by column: 1, 0.3 mM; 2, 0.5 mM; 3, 0.8 mM; 4, 1.0 mM; 5, 2.0 mM. When all additions were made to the tubes, the contents were thoroughly mixed and the tubes placed for 1.5 h. in a 37° incubator. Photographs were made within 2 h. of removing the cells from the incubator.

tographed (Bakerflex IBF silica gel plates, developed in methanol). Concomitantly, this decomposed material loses its ability to agglutinate erythrocytes. Precautions must be taken to avoid exposing solutions of this material to other conditions known to promote deboronation [26]. It is not yet known whether this diboronic acid can cause any of the profound biochemical changes which have been observed to be induced by lectins [27].

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REFERENCES

1. Böeseken, J. (1949) Adv. Carbohyd. Chem. 4, 189-210.
2. Zittle, C.A. (1951) Adv. Enzymol. 12, 493-527.
3. Lorand, J.P., and Edwards, J.O. (1959) J. Org. Chem. 24, 769-774.
4. Ferrier, R.J. (1978) Adv. Carbohyd. Chem. and Biochem. 35, 31-80.
5. Weith, H.L., Wiebers, J.L., and Gilham, P.T. (1970) Biochem. 9, 4396-4401; Rosenberg, M., Wiebers, J.L., and Gilham, P.T. (1972) Biochem. 11, 3623-3628.
6. Ivanova, E.A., Kolodkina, I.I., Grineva, N.J., Tarusova, N.B., Tsilevich, T.L., Gottikh, B.N., and Yurkevich, A.M. (1975) Biochem. (Engl. transl. Biokhimiya) 40, 172-178.
7. Okayama, H., Ueda, K., and Hayaishi, O. (1978) Proc. Nat. Acad. Sci. U.S. 75, 1111-1115.
8. Roe, B.A., Stankiewicz, A.F., and Chen, C.Y. (1977) Nucleic Acid Res. 4, 2191-2204.
9. Schott, H., Rudloff, E., Schmidt, P., Roychoudhury, R., and Kössel, H. (1973) Biochem. 12, 932-938.
10. McCutchan, T.F., Gilham, P.T., and Söll, D. (1975) Nucleic Acid Res. 2, 853-864.
11. Higa, S., Suzuki, T., Hayashi, A., Tsuge, I., and Yamamura, Y. (1977) Anal. Biochem. 77, 18-24.
12. Uziel, M., Smith, L.H., and Taylor, S.A. (1977) Clin. Chem. 22, 1451-1455.
13. Davis, G.E., Suits, R.D., Kuo, K.C., Gehrke, C.W., Waalkes, T.P., and Borek, E. (1977) Clin. Chem. 23, 1427-1435.
14. Moore, E.C., Peterson, D., Yang, L.Y., Yeung, C.Y., and Neff, N.F. (1974) Biochem. 13, 2904-2907.
15. Hageman, J.H., and Kuehn, G.D. (1977) Anal. Biochem. 80, 547-554.
16. Davis, C.W., and Daly, J.W. (1979) J. Cyclic Nucleotide Res. 5, 65-74.
17. Maestas, R.R., Prieto, J.R., Kuehn, G.D., and Hageman, J.H. (1980) J. Chromatog. 189, 225-231.
18. Hanson, R.S., Blicharska, J., and Szulmajster, J. (1964) Biochem. Biophys. Res. Comm. 17, 1-7.
19. Determann, H. (1969) Gel Chromatography, 2nd ed., pp. 16-17, Springer-Verlag, N.Y.
20. Davis-Mancini, K., Lopez, I.P., and Hageman, J.H. (1978) J. Bacteriol. 136, 625-630.
21. Gallop, P.M., and Paz, M.A. (1980) Fed. Proc. 39, 1603.
22. Coutts, I.G.C., Goldschmid, H.R., and Musgrave, O.C. (1970) J. Chem. Soc. (C), 488-493.
23. Damm, H.C., and King, J.W., eds. (1965) Handbook of Clinical Laboratory Data, pp. 346-395, Chemical Rubber, Cleveland.
24. Kabat, E.A., and Mayer, M.M. (1961) Experimental Immunochemistry, 2nd ed., p. 149, C.C. Thomas, Springfield.
25. Johnson, J.R., van Campen, M.G., Jr. and Grummit, O. (1938) J. Am. Chem. Soc. 60, 111-115.
26. Torsell, K. (1964) in: Progress in Boron Chemistry. Steinberg, H., and McCloskey, A.L., eds., Vol. 1, pp. 369-415, Permagon Press, N.Y.
27. Sharon, N., and Lis, H. (1972) Science 177, 949-959; Lis, H., and Sharon, N. (1973) Ann. Rev. Biochem. 42, 541-574.