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Short Communication

Production of Monoclonal and Polyclonal Antibodies against Prostate-Specific Antigen, a Prostate Cancer Serum Marker

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ABSTRACT

The aim of this study was to produce monoclonal and polyclonal antibodies against prostate-specific antigen (PSA), a prostate cancer serum marker. Hyperimmune ICR mice produced polyclonal antibodies (PoAbs) after injection with 0.5 mL of pristane, and were injected with NS-1 myeloma cells 2 weeks later. Hyperimmune Balb/c mice were used for the production of monoclonal antibodies (MAbs). Mice were immunized four times and given a final boost, and their spleen cells were collected and fused with NS-1 myeloma cells under the presence of PEG 1500. The fused cells were then selected in the HAT-RPMIX medium. Anti-PSA antibody-secreting hybridoma cell lines with high titer were cloned by enzyme-linked immunosorbent assay (ELISA) and then subcloned by limiting dilution in 15% fetal bovine serum (FBS) HT-RPMIX medium. Twelve murine hybridoma producing anti-PSA MAbs were obtained and designated C3m1G11, B3m1E5, C3m1E8, C3m1C5, C3m2F4, C3m1F8, C3m2B3, C3m2E6, B3m2B11, B3m2F2, C3m2C7, and C3m2D9. Isotypes of these MAbs were identified as IgG2a heavy chain and κ light chain. Hitrap Protein A column was used for the purification of polyclonal and monoclonal antibodies. The purity analysis of MAb was performed by capillary electrophoresis.

INTRODUCTION

PROSTATE-SPECIFIC ANTIGEN (PSA) is a 32- to 33-kDa single-chain glycoprotein,[1] which has been characterized as a serine protease with restricted chymotrypsin-like specificity belonging to the human kallikrein gene family.[1–3] The protein is secreted by the epithelial cells of the prostate,[4] and levels increase in patients with prostate cancer (PCa).[5] Therefore, immunoassays measuring serum PSA concentrations have been used as screening tests or to facilitate clinical management of PCa.[6] However, increased PSA concentrations in serum also occur in patients with benign prostate hyperplasia (BPH),[7–9] and this reduces the predictive value of PSA measurement for early diagnosis. Normal value of PSA concentration in a healthy adult serum is less than 4 ng/mL.

Different concepts have been used to enhance the specificity of serum PSA measurement without losing sensitivity, including PSA velocity, density, and age-specific reference ranges.[10–12] Furthermore, PSA exists in serum in several forms, of which the most important are the free form of PSA (fPSA), PSA complexed to α-1-antichymotrypsin (PSA-ACT), and PSA complexed to α-2-macroglobulin. The sum of PSA-ACT and fPSA accounts, for most of the total PSA (tPSA) in the serum.[13,14] The observation of significant differences in the proportion of fPSA in patients with PCa when compared with those with BPH or in healthy men originated a new concept based on the ratio of serum fPSA to serum tPSA, the free-to-total PSA ratio (f/tPSA). Recently, better discrimination between PCa and BPH was reported using f/tPSA rather than tPSA, within the diagnostic "gray zone" of tPSA levels of 4–10 ng/mL.[15–18] In a screening population of men with total PSA levels in the "gray zone" about 22% have PCa.[19]

In this paper, monoclonal and polyclonal anti-human PSA antibodies were produced, characterized, and purified. Further-

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more, purities of commercial human PSA and anti-PSA MAbs produced were analyzed. These antibodies will be used for application in the development of tumor marker immunosensors.

MATERIALS AND METHODS

Reagents

1. PSA from human seminal fluid (Chemicon Intl., Temecula, CA) SDS-PAGE ≥90%
2. RPMIX: RPMI 1640 (Seromed, Berlin, Germany) was supplemented with fetal bovine serum (FBS) (Hyclone, Logan, UT) 12%, L-glutamine (200 mM, GibcoBRL, Grand Island, NY) 1%, Pen-Strep (10000 U penicillin G and 10 mg streptomycin/mL solution, 100×, GibcoBRL, Grand Island, NY) 1%, fungizon (250 μg/mL, GibcoBRL, Grand Island, NY) 1% and sodium pyruvate (100 mM, GibcoBRL, Grand Island, NY) 1%
3. Freund's adjuvant (complete and incomplete, GibcoBRL, Grand Island, NY)
4. Peroxidase conjugated goat anti-mouse IgA, IgG, IgM (Capple, Malvern, PA)
5. ABTS (2,2-azino-di-[3-ethyl-benzthiazoline sulfonate] diammonium salt) (Sigma, St. Louis, MO)
6. PEG1500 (polyethylene glycol 1500) (Roche Diagnostics GmbH, Mannheim, Germany)
7. HAT (hypoxanthine 10 mM, thymidine 1.6 mM, aminopterin 1.76 mg/100mL) (GibcoBRL, Grand Island, NY)
8. HT (hypoxanthine 10 mM, thymidine 1.6 mM) (GibcoBRL, Grand Island, NY)
9. Hitrap Protein A column (Amersham Pharmacia Biotech, Inc., Piscataway, NJ)

Materials

The NS-1 myeloma cell line was a gift from Dr. Rong Huay Juang in the Agriculture Chemistry Department of Taiwan University (Taiwan, R.O.C.). Balb/c mice and ICR mice (6–8-week-old, male) were obtained from the Experimental Animal Center of the Medical College of National Taiwan University.

Immunization

All Balb/c and ICR mice were given an initial intraperitoneal (i.p.) immunization with 50 μg of purified antigen in complete Freund's adjuvant (Gibco, Grand Island, NY) and then boosted the antigen in incomplete Freund's adjuvant (Gibco) at 3-week intervals. After 3 months, the ICR mice could produce ascites and the spleens from the Balb/c mice were used in the production of hybridoma cells.[20,21]

Procedure of enzyme-linked immunosorbent assay (ELISA)

Fifty micrograms per milliliter of antigen (human PSA) was adsorbed into a 96-well microtiter plate at 4°C overnight. After coating, the plate was washed twice with phosphate-buffered saline (PBS) (5 mM phosphate buffer, 0.15 M NaCl, pH 7.0). 0.2 mL of gelatin-NET solution (gelatin 0.5%, NaCl 0.15 M, EDTA · 5 mM. Tween 20 0.05%, Tris base 50 mM, pH 8.0) was added for blocking at room temperature. After 1 h, the plate was washed twice with PBST ($NaH_2PO_4 \cdot 2H_2O$ 10 mM, NaCl 0.13 M, Tween 20 0.05%, pH 7.0). 0.1 mL of antibody solution was added to the wells and incubated at 37°C for 30 min, then 4°C for 30 min. After antibody–antigen reaction, the plate was washed three times with PBST, and 0.1 mL of peroxidase conjugated goat anti-mouse antibody was added to the wells and incubated at 37°C for 30 min, then at 4°C for 30 min. After 1 h of incubation, the plate was washed three times with PBST and the enzyme substrate, H_2O_2 and ABTS was added. Absorbance of the colored reaction product at 405 nm was measured by an automated ELISA reader (MR5000, Dynatech).[20,21]

Production of polyclonal antibodies

The hyperimmunized ICR mice were injected with 0.5 mL of pristane (2,6,10,14-tetramethyldecanoic acid). Two weeks later, the mice were injected with 10^6 NS-1 cells. The fluid was tapped

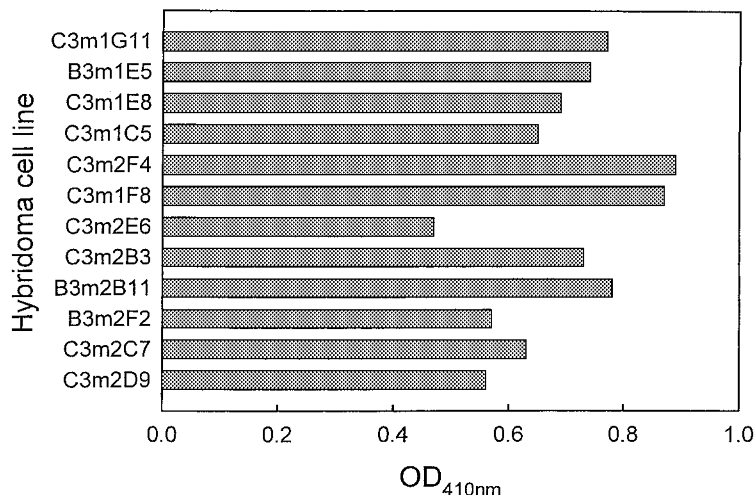


FIG. 1. Selection for anti-human PSA MAb-secreting hybridoma cell lines with high titer.

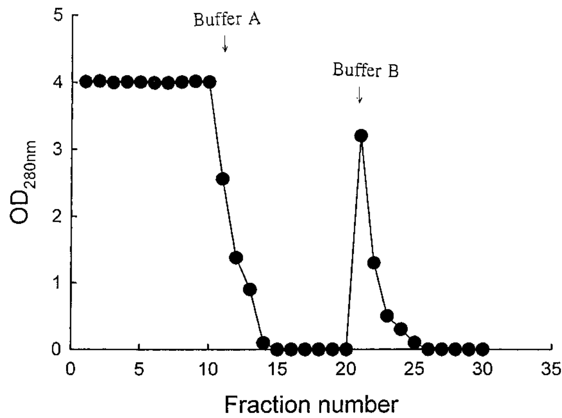


FIG. 2. Affinity chromatogram of Balb/c mice ascites producing monoclonal anti-human PSA antibodies C3m2F4 using Hitrap protein A purification column. 10 mL of the hybridoma ascites was applied into the column. The binding buffer (Buffer A) is a solution containing 0.05 M Tris-HCl, 3 M NaCl (pH 7.8). The elution buffer (Buffer B) is a 0.1 M citrate buffer (pH 5.0). Flow rates of washing and equilibration were 4 mL/min and the rates of sample application and elution were 1 mL/min with 1 mL/fraction.

when the mice were noticeably enlarged, but before the mice had difficulty moving. After centrifugation at 3000 × *g* for 10 min, supernatant was removed and the oil layer discarded.[21]

Production of monoclonal antibodies

At 7 days before fusion and at least 3 weeks after the previous injection, a hyperimmunized mouse was given a final boost of 50 μg of antigen in PBS (pH 7.0). The spleen was then removed, and spleen cells (10^8) were fused with NS-1 myeloma cells (10^7) using PEG 1500. Fused cells were selected in the hypoxanthine, aminopterine, and thymidine (HAT)–RPMIX medium. Anti-PSA antibody-secreting hybridoma cell lines with high titer were cloned by ELISA and then subcloned by limiting dilution in 15% fetal bovine serum (FBS) HT-RPMIX medium.[20,21]

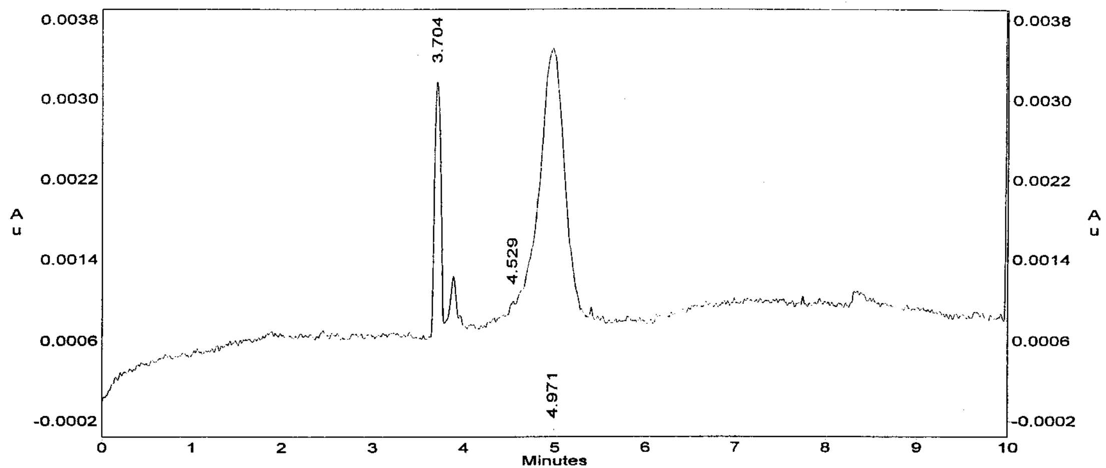
The production of MAbs was scaled up by tissue culture in flasks and ascitic fluid in mice.

Cultures were allowed to grow until the hybridomas died, and the tissue culture supernatants were collected. Debris was removed by centrifugation (1000 × *g*, 10 min) and supernatants were decanted from the cell pellet.[20,21] Supernatant titers were determined by ELISA.

Prime Balb/c mice were injected i.p. with 0.5 mL of pristane or incomplete Freund's adjuvant. After 7–14 days, mice were injected i.p. with 5×10^5–5×10^6 hybridoma cells in 0.5 mL of PBS (pH 7.0). Ascitic fluid began to build up within 1–2 weeks and was tapped when the mouse was noticeably enlarged, but before the mouse had difficulty moving. The fluid was incubated at 37°C for 1 h and maintained at 4°C overnight. After centrifugation at 3000 × *g*, 10 min, supernatant was removed and the oil layer discarded.[20,21]

Monoclonal antibodies characterization

Monoclonal cell culture supernatant (0.1 mL) was added to the ELISA plate, which had adsorbed the antigen. After 1 h of incubation at room temperature, the plate was washed three times with PBST. Eight kinds of isotype goat anti-mouse Ig-peroxidase conjugates were then added to the plate for 1 h of



Channel A Results

Peak	Time	Name	Height (µAU)	Area	Area %	Corrected Area
1	3.70		2485	11689	18.790	2630
2	4.53		113	6545	10.521	1204
3	4.97		2670	43976	70.690	7372
Totals :			5268	62210	100.000	11206

FIG. 3. The purity analysis of commercial human PSA using capillary electrophoresis. PSA from human seminal fluid was purchased from Chemicon Intl. (Temecula, CA).

incubation. The plate was washed three times with PBST and absorbance at 405 nm was measured.

Purification of polyclonal and monoclonal antibodies using Hitrap Protein A column

The sample was pretreated with the ammonium sulfate precipitation. The Hitrap Protein A column was equilibrated with at least two column volumes of starting buffer (20 mM sodium phosphate, pH 7.0). It was then applied to the sample by pumping it into the column, which was washed with the starting buffer for 10 column volumes or until no material appeared in the effluent and eluted with elution buffer (0.1 M citric acid buffer, pH 5.0) 1–3 column volumes. The purified IgG fraction was desalted by dialysis. Flow rates of washing and equilibration were 4 mL/min, and rates of sample application and elution were 2 mL/min.

RESULTS

Ascites formation was induced in hyperimmune ICR mice (serum titer 1:6000) when injected with pristane and then NS-1 myeloma cells after 2 weeks. The highest dilution of the ascites determined by ELISA was 3125-fold. The ascites were purified using Hitrap Protein A column. A single peak of protein fraction (OD_{280nm} = 3.5) was obtained when elution buffer (Buffer B) was applied to the column (data not shown).

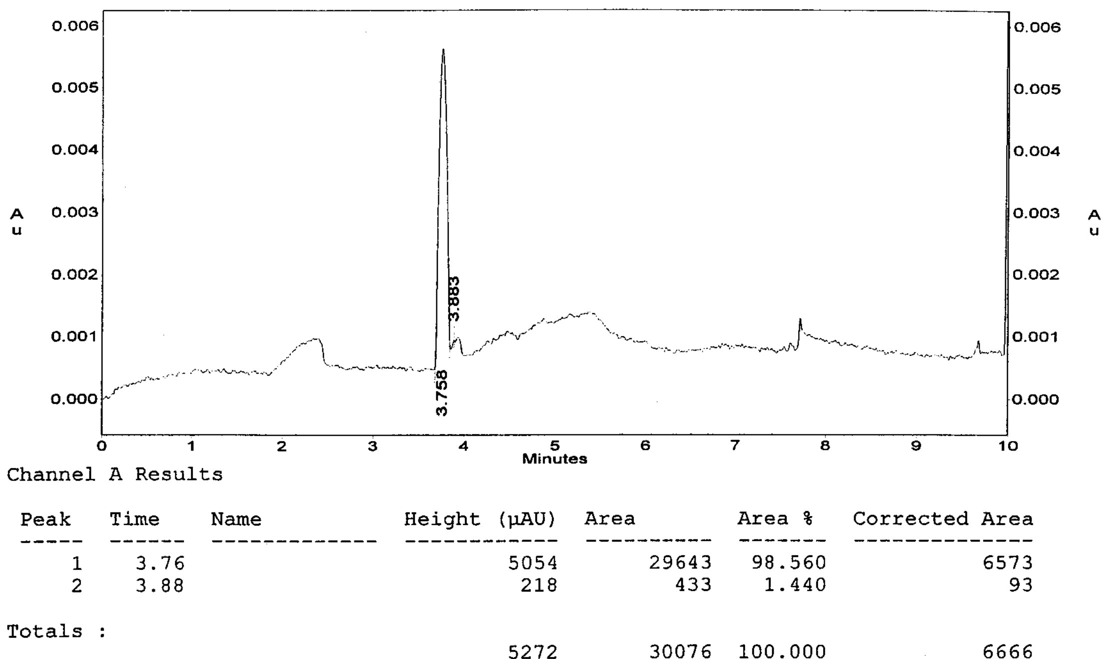
The 12 high-titer MAb-producing hybridoma cell lines selected and designated C3m1G11, B3m1E5, C3m1E8, C3m1C5, C3m2F4, C3m1F8, C3m2B3, C3m2E6, B3m2B11, B3m2F2, C3m2C7, and C3m2D9 are shown in Figure 1. The isotypes of MAbs secreted by the 12 hybridoma cell lines were classified as IgG2a heavy chain and κ light chain using mouse-hybridoma subtyping kit. When high-titer hybridoma (C3m2F4) was injected i.p. into mice, a tumor formed locally or antibody-rich ascites developed. The highest dilution fold of the hybridoma ascites determined by ELISA was 15625-fold.

The ascites containing anti-PSA MAbs C3m2F4 was purified using Hitrap Protein A column. An affinity chromatogram of anti-PSA MAbs C3m2F4 from Balb/c mice ascites using Hitrap A purification column is shown in Figure 2. A single peak of protein fraction (OD_{280nm} = 3.4) was obtained when elution buffer (Buffer B) was applied to the column.

The purities of PSA and MAbs were analyzed by capillary electrophoresis (P/ACE™ System MDQ, Beckman Coulter Taiwan Inc, Taiwan Branch) in the Optoelectronic Biomedicine Center of Medical College of National Taiwan University. Figures 3 and 4 show the purity analysis of the antigen (PSA) and MAb analysis using capillary electrophoresis. The results demonstrated that commercial PSA was not purified, and the MAbs obtained were of high purity.

DISCUSSION

PSA are a type of complete antigen that, when mixed with Freund's adjuvant, can stimulate a good response after injection into mice. A successful fusion procedure brings cells together with an optimal frequency of interactions between the two "parent" cell types. Unfused myeloma cells were dying out as a result of the aminopterin block. Spleen cells were dying out, with the exception of macrophages and/or fibroblasts, which might be establishing themselves and beginning to divide.[20] There appeared to be a correlation between the ap-



Channel A Results

Peak	Time	Name	Height (μAU)	Area	Area %	Corrected Area
1	3.76		5054	29643	98.560	6573
2	3.88		218	433	1.440	93
Totals :			5272	30076	100.000	6666

FIG. 4. The purity analysis of purified anti-PSA MAbs (C3m2F4) by capillary electrophoresis. The purified anti-PSA MAbs (C3m2F4) were prepared using Hitrap Protein A purification column.

pearance of such cells and subsequent good yields of hybrids. The cells were characteristically round with a clear membrane under phase contrast. When the medium in the culture turned yellow, the cultures were screened to determine antibody production and positive colonies by ELISA were selected for expansion and subcloning. Limiting dilution was performed by adding 15% FBS HT-RPMIX medium to replace the conventional method. For maintenance and expansion of MAb-producing hybridoma cell lines, class and subclass were determined on MAb prepared in culture, rather than in mice, to avoid other classes and subclasses, originating from the mouse model.

REFERENCES

1. Lilja H: A kallilrein-like serine protease in prostatic fluid cleaves the predominant seminal vesicle protein. J Clin Invest 1985;76: 1899–1903.
2. Watt KWK, Lee P-J, M'timkulu T, Chan W-P, and Loor R: Human prostate specific antigen: structural and functional similarity with serine protease. Proc Natl Acad Sci USA 1986;83:3166–3170.
3. Lundwall A: Characterization of the gene for prostate-specific antigen, a human glandular kallikrein. Biochem Biophys Res Commun 1989;161:1151–1155.
4. Wang MC, Valenzuela LA, Murphy GP, and Chu TM: Purification of a human prostate specific antigen. Invest Urol 1979;17: 159–163.
5. Wang MC, Papsidero MC, Kuriyama M, Valenzuela LA, Murphy GP, and Chu TM: Prostate antigen: a new potential marker for prostate cancer. Prostate 1981;2:89–96.
6. Ruckle HC, Klee GG, and Oesterling JE: Prostate specific antigen: concepts for staging prostate cancer and monitoring response to therapy. Mayo Clin Pro 1981;69:69–73.
7. Stamey TA, Yang N, Hay AR, McNeal JE, Freiha FS, and Redwine E: Prostate specific antigen as a serum marker for adenocarcinoma of the prostate. N Engl J Med 1987;317:909–916.
8. Hudson MA, Bahnson RR, and Catalona WJ: Clinical use of prostate specific antigen in patients with prostate cancer. J Urol 1989;142:1011–1017.
9. Catalona WJ, Smith DS, Ratliff TL, Dodds KM, Coplen DE, Yuan JJ, Petros JA, and Andriole GL: Measurement of prostate specific antigen in serum as a screening test for prostate cancer. N Engl J Med 1991;324:1156–1161.
10. Carter HB, Pearson JD, Metter J, Brant LJ, Chan DW, Andres R, et al.: Longitudinal evaluation of prostate-specific antigen levels in men with and without prostate disease. JAMA 1992;267:2215–2220.
11. Benson MC, Whang IS, Olsson CA, McMahon DJ, and Cooner WH: The use of prostate specific antigen density to enhance the predictive value of intermediate levels of serum prostate specific antigen. J Urol 1992;147:817–821.
12. Oesterling JE, Jacobsen SJ, Klee GG, et al.: Free, complexed and total serum prostate specific antigen: the establishment of appropriate reference ranges for their concentrations and ratios. J Urol 1995;154:1090–1095.
13. Stenman UH, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K, and Alfthan O: A complex between prostate specific antigen and alpha 1–antichymotrypsin is the major form of prostate specific antigen in serum of patients with prostatic cancer: assay of the complex improves clinical sensitivity for cancer. Cancer Res 1991; 51:222–226.
14. Lilja H, Christensson A, Dahlen U, Matikainen M-T, Nilsson O, Pettersson K, et al.: Prostate specific antigen in serum occurs predominantly in complex with alpha 1–antichymotrypsin. Clin Chem 1991;37:1618–1625.
15. Leinonen J, Lovgren T, Vornanen T, and Stenman UH: Double-label time-resolved immunofluorometric assay of prostate specific antigen and of its complex with alpha 1–antichymotrypsin. Clin Chem 1993;39:2098–2103.
16. Partin AW, Catalona WJ, Southwick PC, Subong EN, Gasior GH, and Chan DW: Analysis of percent free prostate specific antigen (PSA) for prostate cancer detection: influence of total PSA, prostate volume, and age. Urology 1996;48:55–61.
17. Vashi AR, Wojno KJ, Henricks W, et al.: Determination of the "reflex range" and appropriate cutpoints for percent free prostate specific antigen in 413 men referred for prostatic evaluation using the AxSYM system. Urology 1997;49:19–27.
18. Prestigiacomo AF, and Stamey TA: Can free and total prostate specific antigen and prostatic volume distinguish between men with negative and positive systematic ultrasound guided prostate biopsies? J Urol 1997;157:189–194.
19. Catalona WJ, Richie JP, Ahmann FR, et al.: Comparison of digital rectal examination and serum prostate specific antigen in the early detection of prostate cancer: results of a multicenter clinical trial of 6630 men. J Urol 1994;151:1283–1290.
20. Hurrell JGR: *Monoclonal Hybridoma Antibodies: Techniques and Application.* CRC Press, Boca Raton, FL, 1982.
21. Chuang ZH: Studies on sucrose synthetase from rice [Doctoral dissertation]. Graduate Institute of Agriculture Chemistry, National Taiwan University, Taipei, 1985.

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