Variability in Content of the Anti-AIDS Drug Candidate Prostratin in Samoan Populations of *Homalanthus nutans*

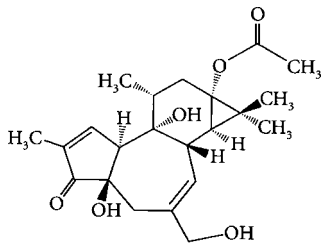
Holly E. Johnson,[†] Sandra A. Banack,[†,‡] and Paul Alan Cox*[,†]

Institute for EthnoMedicine, Box 3464, Jackson, Wyoming 83001, and Department of Biological Science, California State University Fullerton, 800 N. State College, Fullerton, California 92834

Received May 14, 2008

Homalanthus nutans, used by Samoan healers to treat hepatitis, produces the antiviral compound 12-deoxyphorbol 13-acetate, prostratin (**1**). Prostratin is being developed as an adjuvant therapy to clear latent viral reservoirs, the major obstacle to eradication of HIV-AIDS within the human body. A validated reversed-phase HPLC method was developed to assay concentrations of **1** in *H. nutans*. A survey of four distinct populations on two different Samoan islands revealed significant variability in content. The stem tissue (range 0.2−52.6 μg/g **1**), used by healers in indigenous therapies, gave a higher median concentration of prostratin (3.5 μg/g) than root or leaf tissues (2.9 and 2.5 μg/g, respectively). The high variability and skewness of these data indicate that cultivar selection for drug production will be important for this species. The reversed-phase HPLC assay will allow plants to be selected for agricultural development and genetic analysis by identifying those individuals above and below a 95% confidence interval for the median concentration.

Prostratin (**1**) is a nontumor-promoting 12-deoxyphorbol ester that has been shown to inhibit HIV-induced cell death and viral replication in vitro. The antiviral activity of **1** was discovered as a result of ethnobotanical studies on the island of Savai'i, Samoa, where traditional healers use the bark of *Homalanthus nutans* (G. Forst.) Guill. (Euphorbiaceae), a small rain-forest tree called "mamala", to treat hepatitis.[1−3] Permission was granted by healers, village chiefs, and the Samoan government to bring samples of stemwood and healer potions to the Natural Products Branch at the U.S. National Cancer Institute (NCI) for testing. Potent cytoprotective activity against HIV-1 infection was detected in an in vitro tetrazolium-based assay.[4]



Prostratin (**1**)

Bioassay-guided fractionation resulted in the isolation of prostratin (**1**), a tigliane diterpene, from both healer infusions and organic extracts of stem tissue.[4] The compound was not novel, having been previously isolated from *Pimelea prostrata* (J. R. Forst. & G. Forst.) Willd. (Thymelaeaceae).[5] The NCI team found that low concentrations of **1**, from 0.1 to >25 μM, protected T-lymphoblastoid CEM-SS and C-8166 cells from the lethal effects of HIV-1 and inhibited viral replication in these cell lines. Compound **1** also demonstrated cytoprotective activity in the human monocytic cell line U937 and in freshly isolated human monocyte/macrophage cultures.[4,6] Prostratin was found to bind to and activate protein kinase C in vitro in CEM-SS cells.[4] Unlike other known phorbol esters, however, **1** has proved not to be a tumor promoter and has actually been shown to be a potent antitumor promoter.[15,16] Mechanisms involved in the mediation of protein kinase C activity[7−11] as well as other biological activities[12−14] of **1** continue to be studied. Recent studies confirm that **1** is highly potent against HIV infectivity and that this compound has a high in vitro therapeutic index.[17]

Interest in using prostratin (**1**) to help expose latent viral reservoirs within the body was increased when this compound was found to be a potent activator of HIV replication and expression in cells latently infected with HIV-1.[18−22] Latently infected cell reservoirs are currently the major obstacle to viral eradication,[23] even in patients whose plasma viral load is so low as to be undetectable. In an in vitro model, **1** plus interferon has been shown to eliminate 70−80% of latently infected cells.[20] Given the ethnobotanical provenance, its use for centuries among the Samoan people, and its potency in activating latent viral reservoirs, **1** is now a candidate for immediate further research and development for treatment of HIV/AIDS as adjuvant therapy. Hopefully, this can be combined with a protease inhibitor and/or reverse-transcriptase inhibitor, in an effort to expose and eliminate all viral reservoirs in the body.[24]

The AIDS Research Alliance in West Hollywood, California (ARA) has been granted by the NCI the worldwide license for the use of prostratin (**1**) for HIV infection, after first negotiating an equitable return of benefits to the Samoan people as required by NCI.[2] In conjunction with various collaborators, ARA has conducted preliminary pharmacological, pharmacokinetic, and toxicological studies of **1**. The organization is in the process of filing an initial new drug (IND) application to facilitate initiation of phase I human clinical trials with **1** for treatment of HIV/AIDS. The preclinical work has been performed with semisynthetic reagent grade **1**, which is extremely expensive if purchased from a commercial supplier that is not a Good Manufacturing Practice (GMP) facility. ARA is seeking a supply of GMP prostratin for use in further studies and to demonstrate to potential late-stage pharmaceutical development companies that a long-term large-scale supply of active pharmaceutical ingredient (API) can be produced for sales and distribution. In addition, as part of the benefit-sharing agreement with Samoa, ARA has promised to make every possible effort to use *Homalanthus* trees from Samoa as a commercial source. The Samoan government therefore wishes to develop a *Homalanthus* industry in Samoa, based at the village level.

The identification of *H. nutans* plants with extraordinarily high levels of prostratin (**1**) will be vital to the efforts of the Samoan government in selecting genotypes suitable for subsequent cultiva-

* Corresponding author. Tel: (307) 734-1680. Fax: (307) 734-1810. E-mail: paul@ethnomedicine.org.
[†] Institute for EthnoMedicine.
[‡] California State University, Fullerton.

10.1021/np800295m CCC: $40.75
Published on Web 11/14/2008

Table 1. Rank and Content of Prostratin (**1**) in Stem Samples of Samoan *H. nutans*

rank	prostratin (**1**) (μg/g)	population
1	52.69	Saipipi
2	33.65	Falealupo
3	32.45	Saipipi
4	20.55	Falealupo
5	15.09	Tafua
6	14.48	Tafua
7	11.26	Falealupo
8	10.78	Falealupo
9	8.24	Falealupo
10	7.80	Tutuila
11	4.22	Saipipi
12	4.13	Tutuila
13	3.57	Saipipi
14	3.49	Falealupo
15	3.39	Tutuila
16	3.33	Tafua
17	3.18	Saipipi
18	3.14	Tutuila
19	2.98	Tutuila
20	2.28	Tafua
21	2.17	Tafua
22	2.14	Tutuila
23	2.13	Tutuila
24	2.09	Tutuila
25	1.41	Tafua
26	0.58	Saipipi
27	0.20	Tutuila

tion and establishment of an agricultural industry. Similarly, the identification of *H. nutans* plants with extremely low levels of **1** will be important for genetic engineering purposes, which may lead to the discovery of variants or inactive forms of the prostratin gene sequence and the eventual production of low-cost drug through prokaryotic culturing.[25,26] We have developed and validated an assay to rapidly assess **1** content and have evaluated four natural Samoan populations of *H. nutans*. Studies of variability in bioactive molecule concentration potentially could play an increasingly important role in the commercial creation de novo of sustainable pharmaceutical industries based on other pharmacologically active compounds from wild plants.

Significant variability in prostratin (**1**) concentrations was found between *H. nutans* individuals and between populations collected in Samoa. The median concentration of **1** for *H. nutans* stem samples was 3.49 μg/g with a lower 95% confidence limit of 2.28 μg/g and an upper 95% confidence limit of 10.78 μg/g; any plants below or above these limits were regarded as exceptional (Table 1). A Kruskal−Wallis H test allowed rejection of the null hypothesis: H_o = the median prostratin (**1**) concentrations of all populations are equal ($H = 8.31$, df = 3, $p < 0.05$). Chi-square analysis revealed that occurrence of exceptionally high concentration plants was significantly different between populations ($\chi^2 = 7.96$, df = 3, $p < 0.05$) and allowed rejection of the null hypothesis: H_o = the Falealupo population does not have a higher than expected number of exceptionally high-yielding prostratin plants ($\chi^2 = 3.87$, df = 1, $p < 0.05$). Thus, the Falealupo peninsula of Savai'i, Samoa, with the highest median prostratin concentration (11.02 μg/g) and the greatest number of exceptionally high **1**-containing plants, is likely the best population to search for more high-yielding cultivars of *H. nutans*. However, we were unable to reject the null hypothesis for Tafua ($\chi^2 = 1.14$, df = 1, $p = 0.275$) or Tutuila ($\chi^2 = 0.894$, df = 1, $p = 0.330$) populations, having a greater than expected number of exceptionally low **1**-containing plants.

Prostratin (**1**) concentration and diameter of the tree at breast height (DBH) were not significantly correlated (Spearman's rank correlation coefficient, $r_s = -0.236$). A Kruksal−Wallis H test did not allow rejection of the null hypothesis: H_o = the median prostratin concentrations of all plant parts are equal ($H = 2.69$, df = 2). The Spearman rank correlation showed a strong positive correlation between **1** concentrations in the stem and leafy branches ($r_s = 0.783$, $p < 0.05$) as well as in stem and root tissues ($r_s = 0.825$, $p < 0.05$) of the same plant. The general pattern shows higher concentrations of **1** in the stem than in the leafy shoots or in the roots (Table 2), which is consistent with the use of the inner bark by indigenous Samoan healers. The Spearman's rank correlation confirms that plants with exceptionally high stem concentration (such as number 1865) also have relatively higher concentrations in the leafy branches and roots. This suggests that if collection number 1865 were to be developed as a commercial cultivar, both the above-ground and below-ground portions of the plant could be harvested for commercial production of prostratin (**1**).

Table 2. Prostratin (**1**) Content (μg/g) in Various Parts of *H. nutans* Collected in Samoa

sample	root	leafy branches	stem	population
1852	2.77	2.54	4.22	Saipipi
1853	2.87	0.47	3.18	Saipipi
1855	12.82	2.65	52.69	Saipipi
1859	ND	2.32	3.33	Tafua
1860	ND	2.45	2.17	Tafua
1861	ND	0.94	1.41	Tafua
1865	25.03	5.02	33.65	Falealupo
1866	6.65	2.03	3.49	Falealupo
1869	5.95	7.69	11.26	Falealupo
median	2.87	2.45	3.49	

With prostratin (**1**) poised for phase I clinical trials, procuring a consistent long-term supply of GMP prepared compound is essential. The recent elegant synthesis of prostratin by Wender et al.[27] will likely help alleviate this problem, but ARA's license agreement requires that every effort be made to source **1** from Samoa. The prostratin content in various plant parts from four populations of *H. nutans* ranged from 0.20 to 52.69 μg/g, or over a 100-fold difference, which suggests that cultivars should be selected carefully. Knowing the range of variability in the content of **1** will allow the Samoan government, which intends to initiate an agricultural enterprise based on *H. nutans*, to target populations with the best probability of yielding genotypes with exceptionally high prostratin concentration and to ascertain a specific plant's viability as an agricultural cultivar. Development and validation of a robust replicable method of determining concentrations of **1** will be important in creating an initial GMP supply of the molecule for upcoming human clinical trials, as well as in the establishment of a possible resultant *Homalanthus* industry for Samoa.

Variability in prostratin (**1**) content in wild populations is also important for genetic engineering applications. A team led by Keasling at the University of California−Berkeley has developed techniques to transfer genes from different organisms into a prokaryote model to economically produce pharmaceutical molecules of interest.[28] Advances in this field could revolutionize commercial development of bioactive molecules from plants and facilitate efficient inclusion of compounds from indigenous medicine in the modern allopathic drug arsenal. Such approaches are important even though natural product development is at a nadir due to industry decisions to reallocate resources in the search for lead compounds toward combinatorial libraries, an area of clarity in regard to intellectual property. However, it is increasingly clear that combinatorial chemistry has not met its promise. An NCI team found that 61% of the 877 new small molecules introduced as drugs worldwide during 1981−2002 can be traced to natural products; the same team could not identify a single de novo combinatorial compound approved as a drug during the same time frame.[29] Now, with the drug discovery pipeline largely empty, molecules with a long history of human use seem alluring and perhaps the wisdom of natural products drug discovery will be revisited.[30]

The provenance of natural product drugs can often be traced to ethnobotanical research, as was the discovery of the antiviral

properties of prostratin (**1**). However, issues of intellectual property do not present an insurmountable obstacle to commercial development. As shown in the case of **1**, the need to meet the requirements of paragraph 8(j) of the Convention on Biodiversity for equitable sharing of benefits can be resolved ethically.[2,31] A new paradigm for drug research may emerge that incorporates willingness to negotiate fair agreements for natural products collected in foreign countries while meeting practical concerns, such as establishing stable supplies, using technological and methodological innovations.[32] Studies of variability in concentrations of bioactive molecules in plants may play an important role in efficient commercial creation of future phytopharmaceutical industries.

Experimental Section

Plant Material. Samples of stem, root, and leafy branches preserved in 70% ethanol were collected from a population near Saipipi, Savai'i, Samoa. A healer preparation from Falealupo was also collected during the same expedition to Samoa in April 2004. Prostratin (**1**) was detected in each sample, and these materials were used for method development and validation. Another collecting expedition was made to the island of Savai'i, Samoa, in January 2005. Thirty-six samples, consisting of vouchers and ethanol-preserved pharmaceutical grade collections, were taken from three natural populations near the villages of Saipipi, Tafua, and Falealupo. Six trees were sampled from each population, and GPS coordinates, elevation, DBH, height, petiole color, and surrounding vegetation were recorded for each tree sampled. From three plants at each site three different morphological samples were collected: roots (bark + wood), stem (bark + wood), and leafy branches, with fruit and/or flowers if present. Only stem samples were collected from the other three trees at each site for a total of 12 samples per site. Nine stem samples were collected on additional expeditions to the island of Tutuila in American Samoa in May and November of 2005 for a total of 45 samples including 27 stemwood samples. Samples were shipped in vacuum-sealed aluminum vessels in 70% ethanol (except for the Tutuila island collections, which were shipped in 70% 2-propanol). The alcohol fractions were separated and the plant material air-dried in a fume hood. Vouchers were deposited at the herbarium of the California State University Fullerton's Department of Biological Science.

HPLC Analysis. Plant material was finely chopped then pulverized with a coffee grinder, and approximately 1 g of dry sample tissue was suspended in 25 mL of acetone in a 55 °C bath for 10 min. Acetone extracts were filtered using a Whatman no. 4 disk, dried in a Savant AES1000 Speedvac, suspended in 500 μL of 80% ethanol in HPLC grade water, and then filtered with a 0.22 μm Millipore Ultrafree MC centrifugal filter device. Nontarget organics were removed from the alcohol fraction with a Waters Sep-Pak C_{18} cartridge using reversed-phase elution (conditioning: sequential washes of 5 mL of 52% acetonitrile, 5 mL of 26% acetonitrile, and 2.5 mL of 100% HPLC water; loading: 100 μL of sample; separation: 1 mL of HPLC water, 2.5 mL of 26% acetonitrile, and 2.5 mL of 40% acetonitrile); the last fraction was filtered with a 0.22 μm Ultrafree MC centrifugal filter, dried, and suspended in 40 μL of 80% ethanol for injection. The ethanol or 2-propanol fractions and the acetone extract for each of 45 samples were analyzed by HPLC in triplicate to measure prostratin (**1**) concentration in μg per g. Prostratin was separated by reversed-phase elution using a Waters Nova-Pak C_{18} column, 4 μm bead, 300 × 3.9 mm, on a gradient HPLC system (Waters 717 automated injector, Waters 1525 binary solvent delivery system, and Empower data analysis system) at 30 °C. Identification was using a Waters 2487 dual-wavelength UV absorbance detector using an authenticated standard (ICN MP Biomedicals) at 254 nm. Aliquots of 10 μL of each sample were injected, eluted over 15 min with a linear gradient mixing from 32% to 40% acetonitrile using filtered and degassed HPLC grade water (Fisher) and HPLC grade acetonitrile (Sigma Chromasolv), with a flow of 1.0 mL/min.

Method validation was completed in compliance with the specifications in the United States Pharmacopoeia (USP), General Chapter 1225,[33] and Meyer.[34] Ruggedness was evaluated by calculating the precision of biweekly triplicate injections at one concentration during the entire range of the study (RSD 5.21%). Linearity was evaluated by plotting peak area as a function of analyte concentration, and regression analysis was performed: slope = 207.78; intercept = −1468.70; correlation coefficient = 0.9997; residual sum of squares = 716 109 646.17. The limit of detection (LOD) and limit of quantification (LOQ) were determined as 2.5 and 25 pmol, respectively, with a range to 30 nmol. Accuracy (recovery = 96%) was calculated by spiking blank matrix with known amounts of **1** in triplicate at five concentrations. Precision was evaluated using repeatability and intermediate precision. Repeatability was assessed with triplicate injections at five concentrations on two consecutive days (RSD 1.81%). Intermediate precision was calculated biweekly over the range of the study with triplicate injections at three concentrations (RSD 5.87%).

Since these data were not normally distributed, nonparametric statistics were employed. The observed concentrations of **1** were ranked, and 95% confidence limits for the distribution of these concentrations around the median were constructed using eq 8.2.2 in Snedecor and Cochran.[35]

To determine if the median prostratin (**1**) concentrations of all populations were equal, statistical hypotheses were tested using a Kruksal−Wallis H test, the nonparametric analogue of ANOVA. The resultant H statistic was tested for statistical significance at the $p < 0.05$ level using standard χ^2 tables. A chi-square test for independence was employed to ascertain if the occurrence of exceptionally high concentration plants was equal between populations and also to determine if the Falealupo population had a greater than expected number of exceptionally high-yielding prostratin plants. We used a similar chi-square procedure to test the Tafua and Tutuila populations to see if they had a higher than expected number of exceptionally low-yielding **1** plants. Spearman's rank correlation coefficient was calculated to assess the correlation between prostratin concentration and diameter at breast height (DBH). A Kruskal−Wallis H test was employed to establish if **1** was equally distributed throughout plant parts (leafy branches, stem, and root); to determine if plants with high stem concentrations also have high concentrations in the leaf or roots, we calculated a Spearman's rank correlation coefficient and tested for significant correlation at the $p < 0.05$ level.

Acknowledgment. The authors thank S. Murch, T. Buretta, F. Bhakshi, D. Cheng, M. Suazo, J. Schober, and M. Asay for technical support, N. Tavana, G. Tavana, A. Lilo, S. King, and D. Gurr for collection and preparation assistance, the Acacia Foundation for equipment support, K. Farkas for expedition support, and NIH grant R21 TW006608-02 (to P.A.C.) for financial support.

References and Notes

(1) Cox, P. A. *J. Ethnopharmacol.* **1993**, *38*, 181–188.
(2) Cox, P. A. *Pharm. Biol.* **2001**, *39*, 33–40.
(3) Cox, P. A.; Heinrich, M. *Pharm. News* 2001, *8* (3), 55−59.
(4) Gustafson, K. R.; Cardellina, J. H., II; McMahon, J. B.; Gulakowski, R. J.; Ishitoya, J.; Szallasi, Z.; Lewin, N. E.; Blumberg, P. M.; Weislow, O. S.; Beutler, J. A. *J. Med. Chem.* **1992**, *35*, 1978–86.
(5) Zayed, S.; Hafez, A.; Adolf, W.; Hecker, E. *Experientia* **1977**, *33*, 1554–1555.
(6) Tobin, G. J.; Ennis, W. H.; Clanton, D. J.; Gonda, M. A. *Antiviral Res.* **1996**, *33*, 21–31.
(7) Blumberg, P. M.; Acs, G.; Areces, L. B.; Kazanietz, M. G.; Lewin, N. E.; Szallasi, Z. *Prog. Clin. Biol. Res.* **1994**, *387*, 3–19.
(8) Bogi, K.; Lorenzo, P. S.; Szallasi, Z.; Acs, P.; Wagner, G. S.; Blumberg, P. M. *Cancer Res.* **1998**, *58*, 1423–1428.
(9) Nacro, K.; Bienfait, B.; Lee, J.; Han, K. C.; Kang, J. H.; Benzaria, S.; Lewin, N. E.; Bhattacharyya, D. K.; Blumberg, P. M.; Marquez, V. E. *J. Med. Chem.* **2000**, *43*, 921–944.
(10) Hezareh, M.; Moukil, M. A.; Szanto, I.; Pondarzewski, M.; Mouche, S.; Cherix, N.; Brown, S. J.; Carpentier, J. L.; Foti, M. *Antiviral Chem. Chemother.* **2004**, *15*, 207–222.
(11) Trushin, S. A.; Bren, G. D.; Asin, S.; Pennington, K. N.; Paya, C. V.; Badley, A. D. *J. Virol.* **2005**, *79*, 9821–9830.
(12) Kotsonis, P.; Majewski, H. *Br. J. Pharmacol.* **1996**, *119*, 115–125.
(13) Murphy, T. V.; Prountzos, C.; Kotsonis, P.; Iannazzo, L.; Majewski, H. *Eur. J. Pharmacol.* **1999**, *381*, 77–84.
(14) Rullas, J.; Bermejo, M.; Garcia-Perez, J.; Beltan, M.; Gonzalez, N.; Hezareh, M.; Brown, S. J.; Alcami, J. *Antiviral Ther.* **2004**, *9*, 545–554.
(15) Szallasi, Z.; Krsmanovic, L.; Blumberg, P. M. *Cancer Res.* **1993**, *53*, 2507–2512.
(16) Gulakowski, R. J.; McMahon, J. B.; Buckheit, R. W., Jr.; Gustafson, K. R.; Boyd, M. R. *Antiviral Res.* **1997**, *33*, 87–97.
(17) Witvrouw, M.; Pannecouque, C.; Fikkert, V.; Hantson, A.; Van Remoortel, B.; Hezareh, M.; De Clercq, E.; Brown, S. J. *Antiviral Chem. Chemother.* **2003**, *14*, 321–328.

(18) Kulkosky, J.; Culnan, D. M.; Roman, J.; Dornadula, G.; Schnell, M.; Boyd, M. R.; Pomerantz, R. J. *Blood* **2001**, *98*, 3006–3015.
(19) Korin, Y. D.; Brooks, D. G.; Brown, S.; Korotzer, A.; Zack, J. A. *J. Virol.* **2002**, *76*, 8118–8123.
(20) Brooks, D. G.; Hamer, D. H.; Arlen, P. A.; Gao, L.; Bristol, G.; Kitchen, C. M.; Berger, E. A.; Zack, J. A. *Immunity* **2003**, *19*, 413–423.
(21) Biancotto, A.; Grivel, J. C.; Gondois-Rey, F.; Bettendroffer, L.; Vigne, R.; Brown, S.; Margolis, L. B.; Hirsch, I. *J. Virol.* **2004**, *78*, 10507–10515.
(22) Williams, S. A.; Chen, L. F.; Kwon, H.; Fenard, D.; Bisgrove, D.; Verdin, E.; Greene, W. C. *J. Biol. Chem.* **2004**, *279*, 42008–42017.
(23) Brown, H. J.; McBride, W. H.; Zack, J. A.; Sun, R. *Antiviral Ther.* **2005**, *10*, 745–751.
(24) Hezareh, M. *Drug News Perspect.* **2005**, *18*, 496–500.
(25) Chang, M. C.; Keasling, J. D. *Nat. Chem. Biol* **2006**, *2*, 674–681.
(26) Chang, M. C.; Eachus, R. A.; Trieu, W.; Ro, D. K.; Keasling, J. D. *Nat. Chem. Biol.* **2007**, *3*, 274–277.
(27) Wender, P. A.; Kee, J.; Warrington, J. M. *Science* **2008**, *320*, 649–652.
(28) Voigt, C. A.; Keasling, J. D. *Nat. Chem. Biol.* **2005**, *1*, 304–317.
(29) Newman, D. J.; Cragg, G. M.; Snader, K. M. *J. Nat. Prod.* **2003**, *66*, 1022–1037.
(30) Kingston, D. G.; Newman, D. J. *Drugs* **2005**, *8*, 990–992.
(31) Cox, P. A., *Ciba Found Symp*. **1994**, *185*, 25−36;discussion 36−41.
(32) Soejarto, D. D.; Gyllenhaal, C.; Fong, H. H.; Xuan, L. T.; Hiep, N. T.; Hung, N. V.; Bich, T. Q.; Southavong, B.; Sydara, K.; Pezzuto, J. M. *J. Nat. Prod.* **2004**, *67* (2), 294–299.
(33) United States Pharmacopeia. *United States Pharmacopeia*, 27th ed.; United States Pharmacopeial Convention, Inc.: Rockville, MD, 2004.
(34) Meyer V. R. *Practical High-Performance Liquid Chromatography*, 4th ed.; John Wiley & Sons, Chichester, UK, 2004.
(35) Snedecor, G. W.; Cochran W. G. *Statistical Methods*, 8th ed.; Iowa State University Press: Ames, IA, 1989.

NP800295M