Application of Phase-Trafficking Methods to Natural Products Research

Juan J. Araya,[†] Gloria Montenegro,[‡] Lester A. Mitscher,[*,†] and Barbara N. Timmermann[*,†]

Department of Medicinal Chemistry, University of Kansas, Lawrence, Kansas 66045, and Facultad de Agronomía e Ingeniería Forestal, Pontificia Universidad Católica de Chile, Vicuña Mackenna 4860, Santiago, Chile

Received July 7, 2010

A novel simultaneous phase-trafficking approach using spatially separated solid-supported reagents for rapid separation of neutral, basic, and acidic compounds from organic plant extracts with minimum labor is reported. Acidic and basic ion-exchange resins were physically separated into individual sacks ("tea bags") for trapping basic and acidic compounds, respectively, leaving behind in solution neutral components of the natural mixtures. Trapped compounds were then recovered from solid phase by appropriate suspension in acidic or basic solutions. The feasibility of the proposed separation protocol was demonstrated and optimized with an "artificial mixture" of model compounds. In addition, the utility of this methodology was illustrated with the successful separation of the alkaloid skytanthine from *Skytanthus acutus* Meyen and the main catechins and caffeine from *Camellia sinensis* L. (Kuntze). This novel approach offers multiple advantages over traditional extraction methods, as it is not labor intensive, makes use of only small quantities of solvents, produces fractions in adequate quantities for biological assays, and can be easily adapted to field conditions for bioprospecting activities.

In 1963, R. B. Merrifield revolutionized peptide synthesis by introducing solid-phase reagents. This brilliantly simple idea allowed the use of reagents in excess and simplified purification, leading to higher yields and fast isolation.[1] Subsequent elaboration using combinatorial techniques have led to peptide compound libraries of thousands of compounds. Since then, an impressive number of inventive modifications have been introduced in a wide range of fields in academia and industrial laboratories.[2,3] Particularly, organic chemists have taken advantage of specific interactions between small organic molecules and solid-supported reagents (SSR) to achieve quick purification of desired nonpeptide products applying creative phase-switching strategies.[4] Furthermore, the isolation process using solid-phase protocols involves only simple operations of filtration and solvent removal that are suitable for automation and high-throughput applications and has found particular value in combinatorial chemistry laboratories.[5] Despite the multiple advantages of SSR for isolation of small synthetic organic molecules, this method has yet to find application in resolving natural product extracts. Ion-exchange resins have long been used for purification of particular natural products (i.e., quinine[6,7]) at a scale only occasionally used in fractionation schemes. The few examples of applications to natural products research include recovery and concentration of thiamine from rice bran extract,[8] isolation of alkaloids from *Lindelofia achusoides*[9] and *Aconitum septentrionale,*[10] simultaneous determination of phenolics and alkaloids in methanolic extracts of *Gentisia* species,[11] and selective adsorption of tea polyphenols.[12] Generally, the use of exchange resins as column chromatography material in labor-intensive schemes is a common feature in these reports. Wider applications of SSR in natural products research have yet to appear.

The importance of natural products as a source of new therapeutics and as starting materials in medicinal chemistry is undeniable and has been recently reviewed.[13] However, natural products-based drug discovery has become unpopular recently in many industrial laboratories. Particularly, the initial biological activity evaluation of crude extracts has multiple disadvantages, namely, the frequent occurrence of nonselective and nonspecific inhibitors (e.g., polyphenols), the occurrence of a number of chemically diverse components with potentially opposite biological activities, and the low concentration of active metabolites.[14] Therefore, false-positive and false-negative outcomes are possible in both biochemical and cellular screenings, reducing the rate of success and increasing cost. In order to address these difficulties, improved fractionation methods have been developed, including pretreatments to reduce tannins,[15] automated fractionation,[16] single or multiple solid-phase extraction (SPE),[17,18] counter current chromatography,[19] preparative high-pressure liquid chromatography (HPLC), and elaborated applications of complex and costly devices.[20] These methods require either a substantial investment or lengthy and tedious protocols, preventing their implementation, especially in the remote regions of current bioprospecting interest. Consequently, the need for applications that can generate samples conveniently with suitable quality for initial bioassay is of great current interest. Such a method should not only increase the relative concentration of potentially active compounds but also reduce interference from other components in the initial mixture. Also, some additional desirable features would include being fast, inexpensive, environmentally benign, non-labor-intensive, and adaptable to field conditions. To address these needs, we designed and optimized a phase-switching application that takes advantage of weak ion-exchange resins for a simultaneous rapid recovery of neutral, basic, and acidic components from plant crude organic extracts.

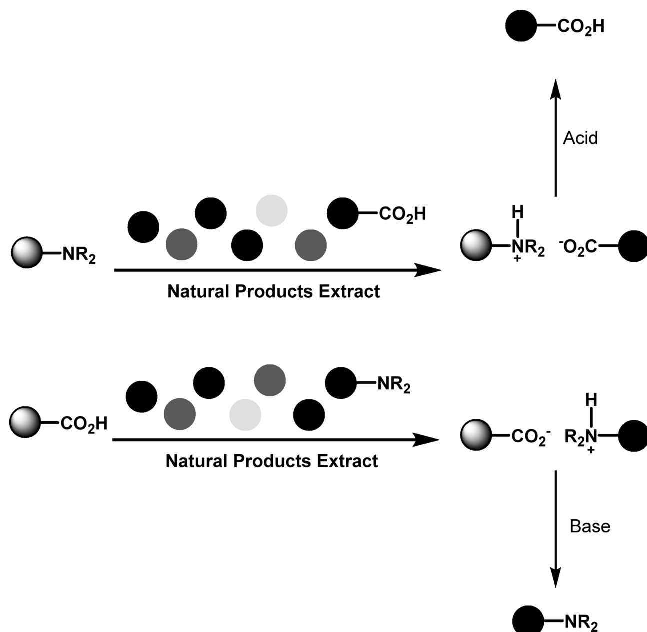
Results and Discussion

Normally, the acid−base character of natural products has allowed selective isolation of compounds based upon their functional groups by using pH manipulation in liquid−liquid partition protocols. However, these more tedious and solvent-demanding conventional solution-phase chemistries can be replaced in principle with simultaneous catch-and-release methodologies using immobilized reagents for natural products extract resolution, as is now commonly done in combinatorial chemistry laboratories.[21] The necessary resins are kept separate from one another by use of porous bags dipped simultaneously in the stirred plant extracts. As illustrated in Scheme 1, groups of acidic and basic compounds can be selectively trapped using an appropriate ion-exchange resin, leaving behind the neutral compounds in solution so that they can each be isolated by simple evaporation. Those operations can, in principle, be adapted to field conditions.

* Corresponding authors. (B.N.T.) Tel: (785)864-4844. Fax: (785)864-5326. E-mail: btimmer@ku.edu. (L.A.M.) Tel: (785)864-4495. Fax: (785)864-5326. E-mail: lmitscher@ku.edu.
[†] University of Kansas, Lawrence.
[‡] Pontificia Universidad Católica de Chile.

10.1021/np100465h © 2010 American Chemical Society and American Society of Pharmacognosy
Published on Web 08/12/2010

Scheme 1. Catch-and-Release Principle of Selective Separation Using Ion-Exchange Resins[a]

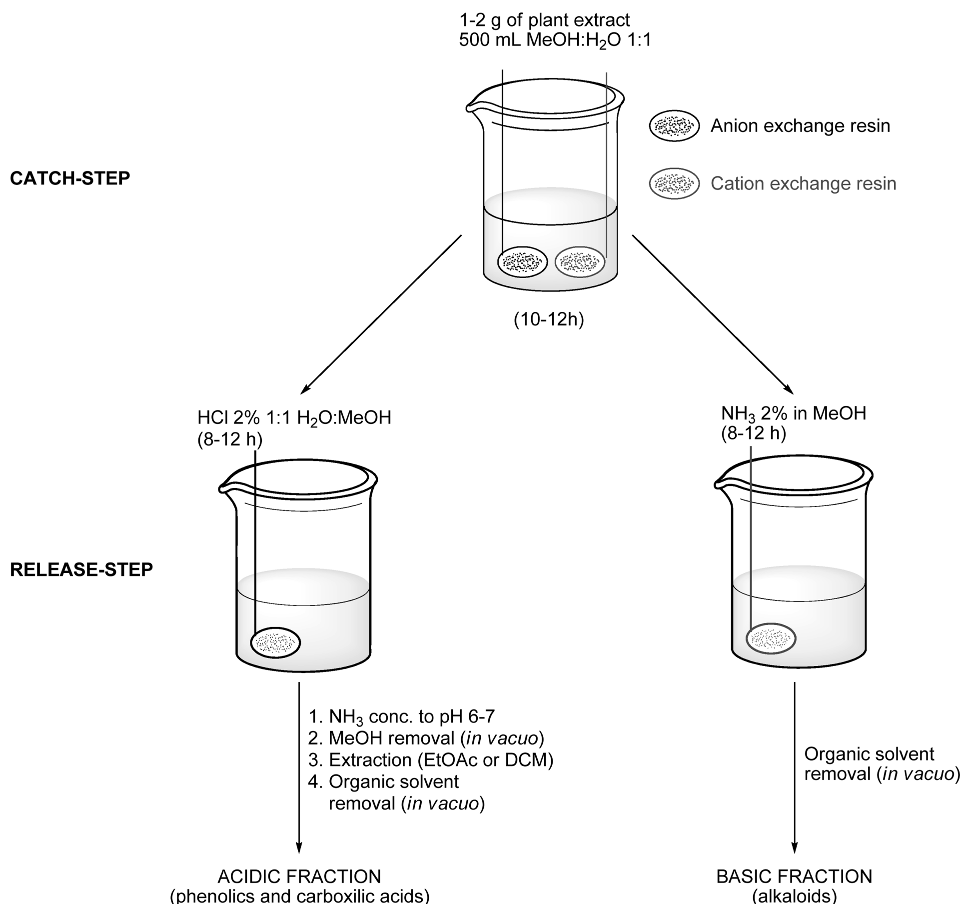


[a] In the first phase the acidic and basic resins are kept spatially separated by employing porous bags. In the second phase the resin bags are withdrawn and separately eluted.

Since spatially separated resins do not interfere with each other's functions,[22] weakly basic and weakly acidic resins can be confined into separate "packets" followed by their joint immersion into a solution of an organic plant extract, allowing for partitioning of its components based upon their acid−base characteristics.[22] In order to work out the conditions necessary to accomplish this, an "artificial extract" was prepared by mixing known amounts of basic, acidic and neutral model compounds (quinine, 3,4,5-trimethoxybenzoic acid, and methyl 3,4,5-trimethoxybenzoate, respectively), and this was subjected to the separation scheme shown in Scheme 2 using the polyacrylic-divinylbenzene resins Dowex MAC-3 (carboxylic acid functional group) and Dowex Marathon WBA (dimethylamino functional group). These resins were chosen because of their large exchange capacities, stability over a wide pH range, and relative ease of regeneration for repeated use.[23,24]

The resins were packed into the tea-filter bags and cleaned according to the manufacturer's guidelines before use. Adsorption of model compounds was followed by HPLC during a 24 h period (Figure 1). Complete sequestration (ca. 98%) of acidic and basic compounds from the extract solution (1−2 g in 500 mL of MeOH−H_2O, 1:1, v/v) was achieved in 8−12 h, while, nonspecific adsorption to the resins of neutral compounds was 14% in the same period using a resin-to-sample ratio of 200:1. A simple saturation experiment showed that a resin-to-sample ratio smaller than 50:1 failed to achieve complete adsorption in a 12 h period (Figure 2). Finally, a change to a water and methanol solvent mixture (data not shown) revealed 1:1 (v/v) as the optimum solvent ratio to use during the trapping step, showing the best balance of solubilization and polarity while reducing the nonselective adsorption of neutral compounds into resins, but still promoting the rapid "switch" of acidic and basic organic compounds from solution onto the respective solid phases.

Scheme 2. General Catch-and-Release Protocol Scheme [a]



[a] The neutral components remain in the original methanol−water solution and are recovered by evaporation.

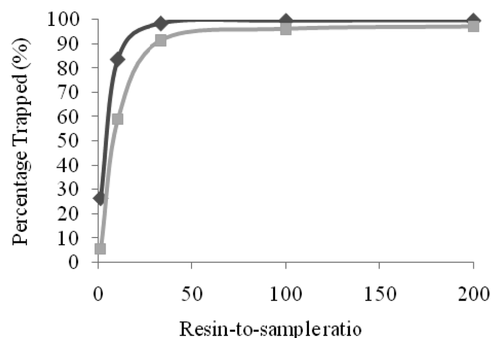


Figure 1. Saturation curves for a 12 h adsorption period of quinine (black diamonds) and 3,4,5-trimethoxybenzoic acid (gray squares).

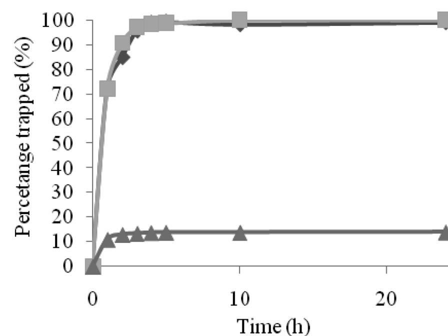


Figure 3. Basic (diamonds), acidic (squares), and neutral (triangles) model compound sequestration in the solid phase in a 24 h period. Percentage is expressed as the fraction of the original concentration ($t = 0$ h) removed from solution.

After removal of the loaded resins from the processed solution, the neutral fraction was simply recovered by removal of solvents under reduced pressure. Acidic and basic fractions were released from the corresponding resins by dipping the bags separately in basic and acid solutions, respectively, under optimized conditions (see Experimental Section). Model compounds were recovered in 75%, 91%, and 98% yields for neutral, basic, and acidic fractions, respectively. In addition, the recovered compounds were highly pure on the basis of HPLC traces (Figure 2). These results demonstrated that the desired selective separation could be achieved with the proposed methodology. Small quantities of the neutral compounds adhered to the resins, presumably due to their lipophilic polymeric backbone (Figure 3). If desired, these compounds could be recovered more completely by washing the resins with pure solvent before release of the ionic contents. The method thus clearly worked efficiently.

Plant alkaloids exhibit a wide range of potent pharmacological activities and are considered very important for drug discovery purposes.[25] Therefore, generation of alkaloid-enriched fractions from plant extracts is very valuable for initial biological screening.

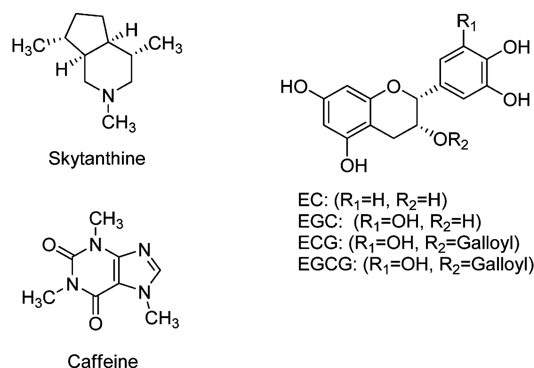


Figure 4. Structures of recovered compounds using catch-and-release approach (from *S. acutus* and *C. sinensis*).

In addition, removal of alkaloids from the plant extract can allow for the evaluation of different types of bioactivities due to other

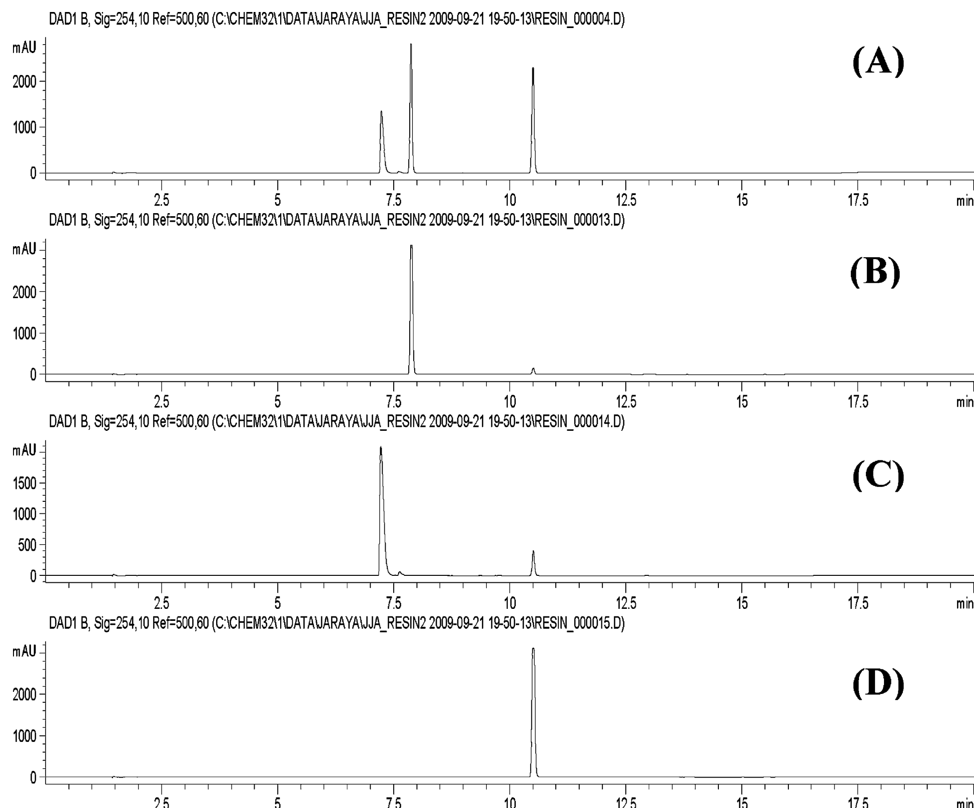


Figure 2. HPLC profile of artificial mixture (A) and recovered fractions: acidic (B), basic (C), and neutral (D).

components possibly masked by the activity of the alkaloids in the original extract.

With the optimized protocol in hand, the plant extracts of *Skytanthus acutus* Meyen (Apocynaceae) and *Camellia sinensis* L. (Kuntze) (Theaceae) were next subjected to the same separation steps in order to examine the utility of the methodology for practical applications to natural product research. The plant *S. acutus* was used as an alkaloid-containing model plant to test and validate our new method in much more complex mixtures. First, the main monoterpene alkaloid present in the methanolic extract of *S. acutus*, skytanthine (Figure 4), was isolated and purified using a traditional isolation scheme as described in the literature.[26] The structure and purity of skytanthine were confirmed by spectroscopic methods (Figures S1 and S2, Supporting Information). The organic extract of *S. acutus* was then submitted to the solid-phase separation scheme. Skytanthine was successfully removed from solution and selectively recovered in the basic fraction as shown in the LCMS traces in Figures S3 and S4 (Supporting Information). Skynthantine lacks a suitable UV chromophore (Figure S3) but is readily detected in the total ion current LCMS traces (Figure S4). From this it is clear that skytanthine, as expected, was concentrated in the acidic resin and was extracted therefrom. In addition, the recovered basic fraction was comparable with the mixture obtained more laboriously by applying a traditional liquid−liquid extraction, by means of LCMS traces (Figures S5 and S6, Supporting Information), and the yield of the alkaloid-rich fraction from the extract (6.1% compared with 5.4% by the traditional partition) was somewhat superior using the new method.

Plant phenolics are a large group of natural products that exhibit a number of useful biological activities and are widely distributed in the plant kingdom, including most of the food plants in the human diet.[27] Green tea catechins have been extensively investigated in the last two decades.[28−31] Also, it is probably the most consumed beverage worldwide.[32] In addition, green tea contains a significant percentage of the purine alkaloid caffeine, making it particularly suitable for the examination of our resin-based separation method. After applying the separation scheme to the green tea organic extract, catechins were analyzed by HPLC. Adsorption of the four main catechins (EC, epicatechin; EGC, epigallocatechin; ECG, epicatechin gallate; and EGCG, epigallocatechin gallate) and caffeine (Figure 4) was followed using HPLC as shown in Figure S7 (Supporting Information). Phenolics were rapidly sequestered from solution in 6 h; however only 50% of the caffeine was removed from solution in this time period. Not surprisingly, the gallate-containg catechins (ECG and EGCG) were adsorbed more rapidly and to a greater extent than the non-gallate-containing counterparts due to their greater acidity. In order to minimize the oxidation of catechins during the recovery stage, the original procedure was slightly modified by using an ultrasound bath for 30 min three times with portions of acidic solution, instead of leaving the sample to shake overnight. The resulting catechin-rich fraction (acidic fraction, 26% yield) clearly showed the presence of four main catechin-related peaks in the HPLC trace (Figure S8, Supporting Information), but only minor amounts of caffeine. Caffeine was now incompletely removed by the acidic resin. The recovered yield (4.4%) was lower, however, when compared with the traditional method (5.5%). Both acidic and basic fractions were comparable to traditional liquid/liquid partition extraction for caffeine ($CHCl_3$ layer, 5.5%) and catechins (EtOAc layer, 30%) by means of HPLC traces.

In summary, a new phase-trafficking approach for acidic, basic, and neutral compound separation from organic plant extracts was developed, validated, and successfully applied not only to artificial mixtures of model compounds but also to crude plant extracts. We envision that this new method could be applied more widely to natural extracts of diverse origin in order to generate better quality samples for initial bioassays. This novel approach offers multiple advantages over traditional extraction methods, as it is not labor intensive, makes use of only small quantities of "green" solvents, is inexpensive, and can be easily adapted to field conditions for bioprospecting; in addition solid-supported reagents can be recycled. Subsequent papers in this series will illustrate further the power of this technique for early-phase examination of plant extracts.

Experimental Section

General Experimental Procedures. Melting points were recorded with an OptiMelt automatic apparatus. IR spectra were obtained with a Thermo Nicolet Avatar 380 FTIR. ^1H NMR, ^{13}C NMR, and two-dimensional spectra were recorded with a Bruker Avance AV-III 500 with a dual carbon/proton cryoprobe. HRMS were conducted with a LCT Premier Waters Corp. apparatus (Milford, MA). Agitations of samples were performed with a New Brunswick Scientific Excella E1 platform shaker.

The following resins and pure compounds were purchased from Sigma-Aldrich (St. Louis, MO): Dowex Marthon WBA anion-exchange resin (batch # 13004PC); Dowex MAC-3 ion-exchange resin (batch # 13228TD); quinine anhydrous (lot code 1375702); 3,4,5-trimethoxybenzoic acid 99% (batch # 05529MH); methyl 3,4,5-trimethoxybenzoate 98% (lot S29247-308). Aromatreu Finum tea filters were purchased from www.cheftools.com.

Plant Material. Aerial parts of *S. acutus* were collected and identified by one of the authors (G.M.), L. Iturriaga, and L. Gonzalez on December 16, 1995, in Caldera, Chile (26°55′ S; 70°67′ W). A voucher specimen has been deposited in the herbarium of the Pontificia Universidad Catolica, Santiago, Chile (coll. no. 0458). *Camellia sinensis* biomass was provided by the Royal Estates Tea Company, a Division of Thomas J. Lipton, Co., Englewood Cliffs, NJ. The green tea blend was labeled "Green Research Standard".

Plant Extraction and Isolation. *S. acutus* and *C. sinensis* biomass were extracted exhaustively with mixtures of MeOH and CH_2Cl_2 (1:1, v/v); then the organic solvents were removed under vacuum to afford the crude organic extract. Each crude extract (2.5 g) was submitted to the general catch-and-release procedure, and the resulting fractions were analyzed by LCMS. In addition, a portion of *S. acutus* extract (10 g) was suspended in water, and 10% HCl was added dropwise to pH < 4 and then extracted three times with CH_2Cl_2. The aqueous layer was then neutralized with NH_3 concentrated to pH > 9 and extracted again with CH_2Cl_2. The resulting alkaloid extract (540 mg, 5.4%) was separated using silica gel SPE (Phenomenex, 20 mm), washing with methanol (100 mL), followed by methanol 5% NH_3, to obtain the crude skytanthine, which was finally purified by recrystallization (CH_2Cl_2−hexanes, 1:1, mp 134.6−135.8 °C). The structure was confirmed by ^1H NMR, ^{13}C NMR, two-dimensional NMR experiments, IR, UV, and HRMS. The data were in agreement with those previously reported in the literature.[26] Finally, for comparison purposes, the green tea extract (2.5 g) was suspended in water and extracted successively with $CHCl_3$ and EtOAc to generate caffeine- and catechin-rich fractions, respectively.

General Catch-and-Release Procedure. Plant organic extract (2.5 g) was suspended in 500 mL of MeOH−H_2O (1:1, v/v). Prewashed tea bags containing 20 g of Dowex Marathon WBA anion-exchange resin and 20 g of Dowex MAC-3 cation-exchange resin were dipped into the solution and left shaking at 25 rpm overnight (8−10 h). Tea bags were then removed and washed with MeOH twice, then submitted to recovery conditions. Anion-exchange resin was immersed into 500 mL of 2% HCl (v/v in MeOH−H_2O, 1:1) and left overnight under agitation (25 rpm); then the HCl was neutralized with NH_4OH to pH 6−7, the MeOH removed under reduced pressure, and the aqueous phase extracted three times with EtOAc or CH_2Cl_2. Removal of the separated organic extract afforded the phenolic/acidic fraction upon evaporation. Cation-exchange resin was immersed into 500 mL of NH_3 2% (v/v in MeOH) and left overnight under agitation, and the resulting solution was concentrated under reduced pressure to afford the alkaloidal fraction. The original working solution was also concentrated under reduced pressure to yield the neutral fraction.

Artificial Extract Preparation and Separation. Approximately 100 mg of each of the model compounds (quinine, 3,4,5-trimethoxybenzoic acid, and methyl 3,4,5-trimethoxybenzoate) was weighed with an analytical balance and dissolved in 500 mL of a mixture of MeOH and H_2O (1:1) and was submitted to the general catch-and-release procedure. During the first 6 h, every hour a 1.0 mL sample was taken and analyzed using HPLC; then additional aliquots were taken after

10 and 24 h. Concentrations were determined by interpolation from a calibration curve prepared for each compound by appropriate dilution of a mother solution of 20 mg/mL to final concentrations of 0.1 to 1.0 mg/mL.

HPLC/MS^n Analyses. The online HPLC/MS^n analyses of extracts and fractions were performed using an Agilent 1200 Series liquid chromatography system coupled to the Agilent IonTrap LCMS 6310 mass spectrometer. The positive ion ESIMS experimental conditions were as follows: HV capillary voltage, 3.5 kV; drying temperature, 350 °C; drying gas, 12.0 L/min; nebulizer, 15 psi; and capillary exit voltage, 124.8 V. The Frag Ampl was set to 1.0 V, and the smart fragmentation function was used (Smart Frag Ampl was 30−200%). HPLC separations were done using an Agilent Eclipse XDB-C18 column (5 μm, 4.6 × 150 mm), and the flow rate was 1.0 mL/min (approximately 80 bar). The mobile phase for *S. acutus* samples was a linear gradient of acetonitrile and water from 10:90 (v/v) ($t = 0$ min) to 100:0 ($t = 25$ min), then 100:0 until ($t = 30$ min), and finally 10:90 during 10 min ($t = 40$ min) for recovery. On the other hand, the mobile-phase gradient program for *C. sinensis* samples was acetonitrile and 5 mM formic acid [5:95 (v/v) ($t = 0$ min), 15:85 ($t = 15$ min), 100:0 ($t = 35$ min)], wash for 5 min, and finally recovery to 5:95 ($t = 50$ min). All samples were dissolved in the mobile phase to a concentration of 1.0 mg/mL and filtered using 13 mm filters with 0.45 μm PTFE membranes (VWR). The injection volume was 25 μL.

Acknowledgment. The authors are thankful to Luis Gonzalez and Liliana Iturriaga for the collection and identification of *S. acutus*. The field work was supported by the ICBG grant 5 UO1 TW000316 from the National Institutes of Health (NIH) to B.N.T., whereas the chemical work was supported by the University of Kansas Center for Research project 2506014-910/099 to B.N.T. J.A. thanks the LASPAU-Fulbright program and the University of Costa Rica for financial support. The contents are solely the responsibilities of the authors and do not necessarily represent the official views of the NIH.

Supporting Information Available: ^{1}H and ^{13}C NMR of skytanthine. HPLC and LCMS traces of *S. acutus* and *C. sinensis* extracts and fractions. Adsorption curve of green tea catechins in solid-phase reagent. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149–2154.
(2) Cano, M.; Balasubramanian, S. *Drugs Future* **2003**, *28*, 659–678.
(3) Alexandratos, S. D. *Ind. Eng. Chem. Res.* **2009**, *48*, 388–398.
(4) Solinas, A.; Taddei, M. *Synthesis-Stuttgart* **2007**, *16*, 2409–2453.
(5) Kennedy, J. P.; Williams, L.; Bridges, T. M.; Daniels, R. N.; Weaver, D.; Lindsley, C. W. *J. Comb. Chem.* **2008**, *10*, 345–354.
(6) Applezweig, N.; Ronzone, S. E. *Ind. Eng. Chem.* **1946**, *38*, 576–579.
(7) Applezweig, N. *J. Am. Chem. Soc.* **1944**, *66*, 1990–1990.
(8) Herr, D. S. *Ind. Eng. Chem.* **1945**, *37*, 631–634.
(9) Babaev, B.; Abdullaev, N. P.; Shakirov, T. T. *Chem. Nat. Compd.* **1976**, *12*, 109–110.
(10) Sayed, H. M.; Desai, H. K.; Ross, S. A.; Pelletier, S. W.; Aasen, A. J. *J. Nat. Prod.* **1992**, *55*, 1595–1606.
(11) Vanrensen, I.; Veit, M. *Phytochem. Anal.* **1995**, *6*, 121–124.
(12) Zhao, R. Y.; Yan, Y.; Li, M. X.; Yan, H. S. *React. Funct. Polym.* **2008**, *68*, 768–774.
(13) Cragg, G. M.; Grothaus, P. G.; Newman, D. J. *Chem. Rev.* **2009**, *109*, 3012–3043.
(14) Li, J. W. H.; Vederas, J. C. *Science* **2009**, *325*, 161–165.
(15) Wall, M. E.; Wani, M. C.; Brown, D. M.; Fullas, F.; Olwald, J. B.; Josephson, F. F.; Thornton, N. M.; Pezzuto, J. M.; Beecher, C. W. W.; Farnsworth, N. R.; Cordell, G. A.; Kinghorn, A. D. *Phytomedicine* **1996**, *3*, 281–285.
(16) Tu, Y.; Jeffries, C.; Ruan, H.; Nelson, C.; Smithson, D.; Shelat, A. A.; Brown, K. M.; Li, X.-C.; Hester, J. P.; Smillie, T.; Khan, I. A.; Walker, L.; Guy, K.; Yan, B. *J. Nat. Prod.* **2010**, *73*, 751–754.
(17) Thiericke, R. *J. Autom. Methods Manage. Chem.* **2000**, *22*, 149–157.
(18) Månsson, M.; Phipps, R. K.; Gram, L.; Munro, M. H. G.; Larsen, T. O.; Nielsen, K. F. *J. Nat. Prod.* **2010**, *73*, 1126–1132.
(19) Wu, S.; Yang, L.; Gao, Y.; Liu, X.; Liu, F. *J. Chromatogr. A* **2008**, *1180*, 99–107.
(20) Tu, Y.; Jeffries, C.; Ruan, H.; Nelson, C.; Smithson, D.; Shelat, A. A.; Brown, K. M.; Li, X.-C.; Hester, J. P.; Smillie, T.; Khan, I. A.; Walker, L.; Guy, K.; Yan, B. *J. Nat. Prod.* **2010**, .
(21) Flynn, D. L. *Med. Res. Rev.* **1999**, *19*, 408–431.
(22) Houghten, R. A. *PNAS USA* **1985**, *82*, 5131–5135.
(23) Dowex Marathon WBA Engineering Information. http://www.dow.com/liquidseps/prod/dx_mar_wba.htm (accessed 03−31−2010).
(24) Dowex Mac-3 Engineering Information. http://www.dow.com/liquidseps/prod/dx_mac3.htm (accessed 03−31−2010).
(25) Cordell, G. A.; Quinn-Beattie, M. L.; Farnsworth, N. R. *Phytother. Res.* **2001**, *15*, 183–205.
(26) Djerassi, C.; Shamma, M.; Kutney, J. P. *Tetrahedron* **1962**, *18*, 183.
(27) Crozier, A.; Jaganath, I. B.; Clifford, M. N. *Nat. Prod. Rep.* **2009**, *26*, 1001–1043.
(28) Mitscher, L. A.; Jung, M.; Shankel, D.; Dou, J. H.; Steele, L.; Pillai, S. P. *Med. Res. Rev.* **1997**, *17*, 327–365.
(29) Gensler, H. L.; Timmermann, B. N.; Valcic, S.; Wachter, G. A.; Dorr, R.; Dvorakova, K.; Alberts, D. S. *Nut. Cancer* **1996**, *26*, 325–335.
(30) Valcic, S.; Timmermann, B. N.; Alberts, D. S.; Wachter, G. A.; Krutzsch, M.; Wymer, J.; Guillen, J. M. *Anti-Cancer Drugs* **1996**, *7*, 461–468.
(31) Valcic, S.; Burr, J. A.; Timmermann, B. N.; Liebler, D. C. *Chem. Res. Toxicol.* **2000**, *13*, 801–810.
(32) Pharn-Huy, L. A. N.; He, H.; Phamhuy, C. *J. Food Agric. Environ.* **2008**, *6*, 6–13.

NP100465H