Solid-Phase Synthesis of Large Tetrahydroisoquinolinone Arrays by Two Different Approaches

Michal Lebl,[1] Viktor Krchňák,[2] Georges Ibrahim,[1] Jaylynn Pires,[1] Christine Burger,[3] Yidong Ni,[4] Yuewu Chen,[3] David Podue,[3] Petr Mudra,[5] Vit Pokorny,[5] Pavel Poncar,[5] Karel Zenisek[5]

[1]Spyder Instruments Inc., 9390 Towne Centre Drive, Suite 200, San Diego, CA 92121, USA

[2]SIDDCO, P040 S. Rita Road, Bldg. 40, Tucson, AZ 85747, USA

[3]Trega Biosciences Inc., 9880 Campus Point Drive, San Diego, CA 92121, USA

[4]Glaxo-Wellcome, 5 Morse Drive, PO Box 13398, Research Traingle Park, NC 277c, USA

[5]Institute of Organic Chemistry and Biochemistry, Flemingovo 2, 166 10, Praha 6, Czech Republic

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Abstract: We have developed two new methods for the synthesis of large arrays of small organic molecules and employed them in the synthesis of tetrahydroisoquinolinones. The first method, using a combination of "tea-bag" synthesis and synthesis in microtiterplates with "surface suction" separation of solid and liquid phase, was applied in the production of a library of 30,816 compounds for general screening. The second method, using "tilted centrifugation", was employed for the rapid synthesis of an array of 768 compounds for "lead explosion".

Key words: combinatorial chemistry, parallel synthesis, surface suction, centrifugal synthesizer, gaseous HF

Introduction

The introduction of combinatorial techniques (for reviews see e.g.[1]) into drug discovery process resulted in "rediscovery" of solid phase synthesis for preparation of organic compounds. Solid phase synthesis[2,3] became routine for the preparation of peptides and oligonucleotides, and numerous automatic synthesizers exist for completely unattended preparation of large numbers of very long sequences. But it was the publication of Ellman in 1992,[4] which triggered the world's attention to solid phase synthesis of "other" organic molecules. (Surprisingly enough, the earlier work of Leznoff,[5] Patchornik,[6] Camps,[7] and others was not noticed.) Solid-phase synthesis of small organic molecules requires new methods for automation of synthetic processes. Even though solid phase synthesis is optimal for automation, since the complicating factor of the unique behavior of different organic molecules is replaced by the predictable behavior of the solid support, peptide and oligonucleotide synthesizers were not ready to perform the required job. First, they were designed for very well defined chemistries and sets of building blocks, and secondly, they were optimized for individual syntheses of long sequences. The requirements of combinatorial chemistry are different: to synthesize large numbers of relatively small molecules using very varied sets of chemistries and building blocks.

One of the basic problems in the design of solid phase synthesizers is the parallel separation of liquid and solid phases. All of commercial solid phase synthesizers utilize filtration as the principle for separation of solid and liquid phase (for reviews see e.g.[8,9]). Filtration can lead to significant complications, especially in the case of multiple synthesizers, since the clogging of one vessel can result in overflowing of this particular vessel during the next solvent addition and distribution of the solid support from this vessel into neighboring ones. Understanding that the filtration is one of the major obstacles in the application of solid phase techniques to large throughput parallel synthesis, we have developed a process referred to as the “surface suction” method. This method does not require the use of any porous material for separation of liquid and solid phases.[10]

The simplest way to remove a liquid is to immerse a needle in it and suck the liquid out. We connected the needle to an evacuated waste container before the needle touched the liquid surface. The needle was then slowly lowered against the surface so the liquid was shaved from the surface without disturbing the settled resin beads. Therefore we could go very close to the layer of sedimented particles without removing them from the mixture. Obviously, this method requires that the solid phase sediments in the washing step.

We have built the robotic station, which can process up to 72 microtiterplates in one batch.[9] However, the described “split only” technique is applicable also for the manual synthesis of sizable libraries (>10,000 compounds). Synthesis can start for example in Domino Blocks,[11] followed by the surface suction method in plates.

Instruments available on the market today are relatively complicated and expensive. An instrument that would be rather simple, and therefore inexpensive, and which would allow each chemist to synthesize hundreds or thousands of compounds would be welcomed by a number of medicinal chemists. Such an instrument would be used for the deconvolution of active compounds from biologically active mixtures, synthesis of arrays of compounds for general screening, or for compound optimization, so called “lead explosion”.

This article describes two approaches that we have used at Trega Biosciences for the preparation of large arrays of small organic molecules.

Results and Discussion

Both methods that we used for the synthesis of combinatorial arrays of compounds on solid phase avoided the use of porous material for the separation of liquid and solid phase.

Surface Suction Principle

We have built a robotic station that uses the surface suction principle. The robotic station is very simple intelligent gripper capable of picking up a deep well microtiterplate, transferring it under the array of 96 flat-end stainless steel needles, lifting it against this array while suction through the needles is applied, lowering it, transferring it under an array of peek needles through which the washing solvent is added, and placing it back on the table. This machine can use an array of solvents for the washings and can work under inert atmosphere. The inert atmosphere (which was not used in the described synthesis) requires a constant supply of inert gas into the envelope covering the machine. Application of continuous suction would use excessive amounts of the inert gases. We have solved this problem by applying the suction only when needed for the liquid removal. This solution seems obvious, but practical realization required the application of an intermediate evacuated container with the volume only slightly bigger than the expected amount of solvent removed from a single microtiterplate. Operation of this flask is best described by following it in one cycle (see Figure 1). A constantly running vacuum pump is attached to this flask through a solenoid valve S1. Another line attaches it to compressed nitrogen (via solenoid valve S2). The flask is also attached to the 96 channel needle suction manifold (via solenoid valve S3) and the waste barrel (via solenoid valve S4). This last line can be branched for separation of solvents into several waste lines. Valve S1 opens at least three seconds before the arrival of a microtiterplate under the suction manifold. When the microtiterplate reaches the needle array, valve S3 opens and stays open until the microtiterplate reaches the uppermost position. After another three seconds valves S3 and S1 close, and valves S2 and S4 open. Liquid collected from the microtiterplate in the intermediate flask is transferred to the waste barrel, valves S2 and S4 close and valve S1 opens to prepare for the suction from the next microtiterplate. In this way the consumption of inert atmosphere from the enclosed compartment can be minimized and at the same time, the large waste barrel does not have to be evacuated.

The described procedure can process a large number of microtiterplates in parallel, but the procedure is limited basically to the parallel washing. To achieve really high throughput synthesis, we decided to combine the power of the parallel robotic processing of microtiterplates with the power of "tea-bag" technology[12] for solid phase synthesis. Manual solid phase synthesis in "tea-bags" is very flexible and a wide span of conditions can be used in the first several steps of the synthesis. The reaction conditions are limited only by the properties of the polypropylene mesh from which the "tea-bags" are constructed. Up to a thousand "tea-bags" can be handled simultaneously by one chemist and they can be reacted in up to fifty reaction vessels simultaneously. In this way up to a thousand of intermediates can be prepared and the resin from each "tea-

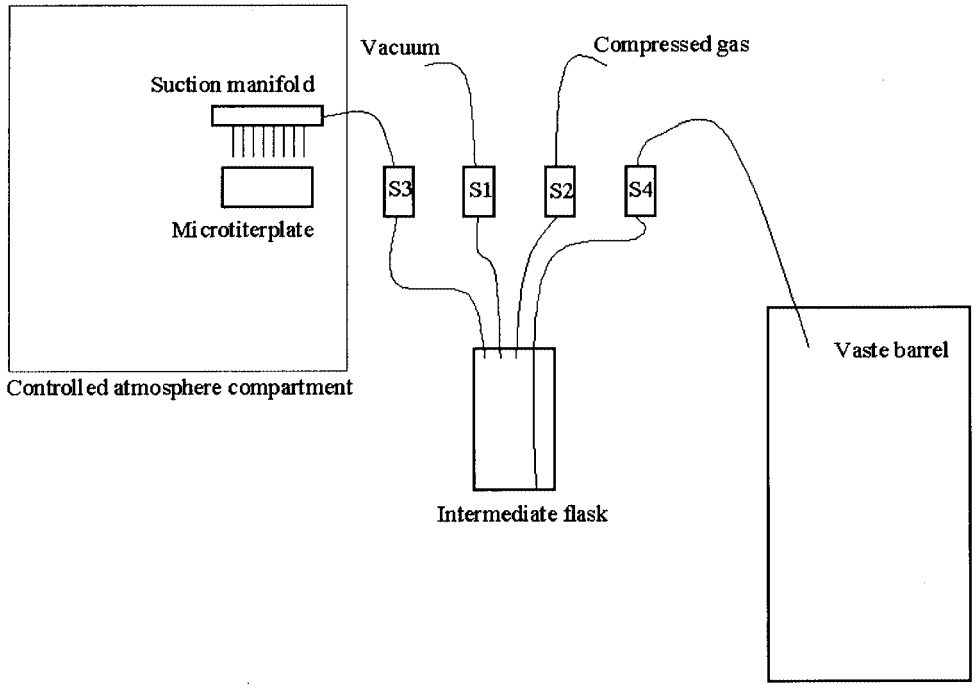

Figure 1 Scheme of liquid transfer conserving inert atmosphere in the robotic envelope

bag" can be distributed into a microtiterplate's wells. Before distribution, a sample from each bag is cleaved and the product is analyzed by LC/MS. Only bags containing the expected material in purity better than 85% (evaporative light scattering or UV detection) are used for the continuation of the synthesis. Individual wells of the microtiterplate then receive a different building block and/or reagent by simultaneous pipetting (TomTec Quadra 96) from a pre-prepared "master plate" in which an array of reactants was assembled. In this way, each intermediate is used for the synthesis of up to 96 individual compounds ("bag explosion" – 1,000 bags can result in 96,000 individual compounds). The limitation of this approach is the necessity of "process friendliness" of the last step of the synthesis (relatively stable reagents, temperature range from room temperature up to 80 degrees). The resin in the wells of the microtiterplate is then incubated at the appropriate temperature and washed by the application of the surface suction technique. The addition of building blocks and reagents, and incubation can be repeated as many times as needed. After finishing the synthesis and final wash, the plates are dried in vacuum and placed into polypropylene chambers (Figure 2) where they are exposed to gaseous HF at room temperature for 2 hours. Gaseous HF is removed by nitrogen blowing, and plates are transferred into desiccators for overnight evacuation. The plates are then transferred to the platform of the Multiprobe 208 (Packard Canberra) and the product is extracted by repeated exposure to neat acetic acid. Acetic acid is a powerful extractant and allows for simultaneous removal by lyophilization. Other solvents can be used for extraction, but the only alternative simultaneous way of solvent removal is vacuum centrifugation (GeneVac). Every compound from the production is analyzed by direct injection into a mass spectrometer (one injection every 10 seconds) and 12% of the library is evaluated by HPLC with gradient elution.

Figure 2 Polypropylene chambers used for cleavage of compounds from benzhydrylamine resin by exposure to gaseous HF. The window is made of polymethacrylate covered with polypropylene foil.

Trega has synthesized a number of libraries of a size of 5,000 to 60,000 by using this technique and we present here (see experimental section) the synthesis of an array of 30,816 tetrahydroisoquinolinones as an example. The synthetic scheme (Figure 3) developed earlier for the synthesis of mixture libraries in tea bags [13] was modified for the "surface suction" technique. In the first step of library synthesis we have employed nucleophilic substitution of bromine in bromoacetic acid attached to solid support by an array of primary amines. This removed the limitation imposed by the availability of appropriate amino acids used as the first building block in the original library design. The purities of the prepared compounds are given in the Table. From 780 bags 90 did not pass the quality control in the first stage (after nucleophilic substitution and acylation by beta alanine, 90% cut-off at quality control by ELSD HPLC) and additional 48 bags were removed before "bags explosion" into microtiterplates (85% cut-off). From finally processed 321 plates (30,816 compounds), 58 plates (18%) did not pass the final quality control criteria (>75% of compounds in a plate must have correct molecular ion of more than 10% intensity of a base peak at MS evaluation, and the sample from the plate, one row, must have purity by ELSD HPLC better than 75%). All 30,816 compounds were analyzed by direct infusion mass spectroscopy. In 1,321 compounds the expected molecular peak was not found and in 3,722 cases the expected molecular peak was found, but the intensity was lower than 10% of the base peak. A sample of 3,158 compounds was analysed by ELSD HPLC and the average purity was found to be 83.99%. The distribution of average (percentage of wells with correct molecular weight and average purity of 12% of wells according to ELSD HPLC) analytical data over all plates is given in Figure 4. The average amount of compound synthesized in each well was 10.38 mg. Since the synthesis was performed in all 96 wells of the plate, the compounds were transferred into the daughter plates in different format (80 wells per plate) by automated pipetting (Multiprobe 208, Packard Canberra) and delivered for biological evaluation.

Tilted Centrifugation Technique

The surface suction technique still does not allow for processing of an unlimited number of reaction vessels simultaneously, the number of processed vessels depends on the number of needles performing the suction. With 72 plates on the robotic surface, only 6,912 compounds can be processed in one batch.

But there is a simpler way for simultaneous processing of hundreds or thousands of reaction vessels. We call this new technique "tilted centrifugation".[14,15] The principle of tilted centrifugation is shown in Figure 5. A resin-containing vessel is attached in the tilted position at the perimeter of the centrifugal plate and spun. Resin, which has sedimented at the bottom of the vessel, does not remain at the bottom of the flask. As the surface of liquid supernatant moves, the solid support layer moves as well. If the speed of rotation is increased, the centrifugal force created by

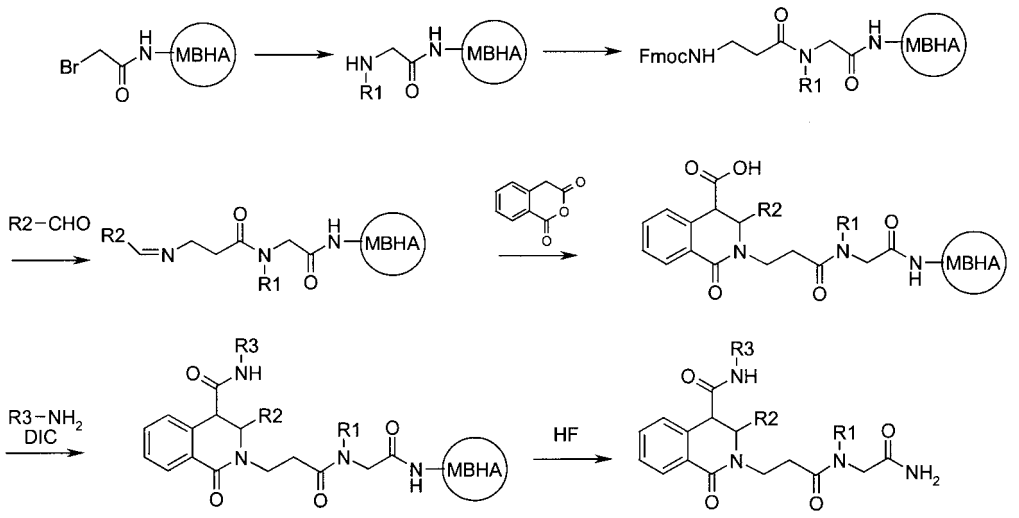

Figure 3 Synthetic scheme for library of tetrahydroisoquinolinones

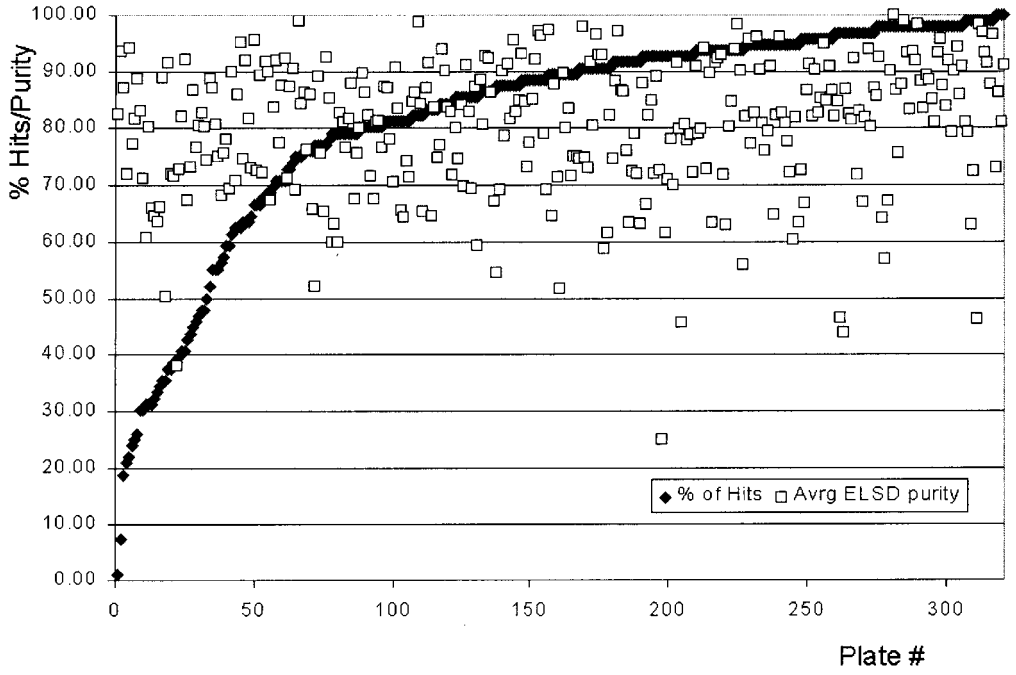

Figure 4 Distribution of analytical data for 321 microtiterplates

rotation (which depends on the radius of rotation and the speed) combines with gravitation and the resulting force causes the liquid surface to stabilize at an angle perpendicular to the resulting force vector. At the ratio of relative centrifugal force (RCF) to g of 3, the angle of the liquid surface will be about 61 degrees. If the speed is increased so that the ratio of these forces is more than 50, the situation is close to the RCF of infinity – therefore, the liquid (and resin layer) angle will be close to 90 degrees. The pocket created by the tilt now allows only solid phase to remain in the pocket and all of the liquid is expelled. The pocket can be created in a vessel of basically any shape - flat bottom, U bottom, or V bottom vessel, as well as in an array of vessels, e.g. in the commonly used microtiterplates.

The situation of wells in microtiterplates placed on the perimeter of the centrifuge depends on the distance of the individual well from the axis of rotation. The volume of the "pocket" created by centrifugation in the wells closer to the axis is bigger than the volume of the "pocket" created in the wells more distant from the center of rotation. However, the volume of the pocket is not as important as the ratio of the volumes of pockets in different wells of the microtiterplate. This ratio depends on the dimension of the centrifugal rotor, the speed of the rotation, and the tilt

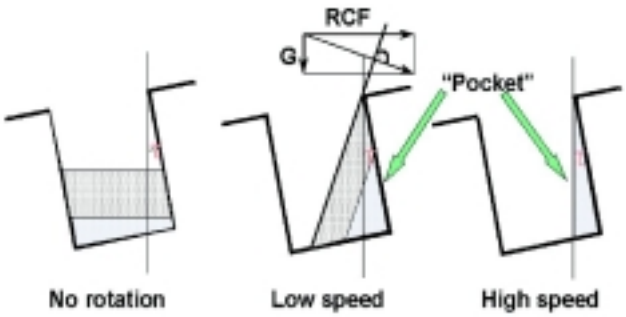

Figure 5 Principle of "tilted centrifugation"

of the plate. We decided to work with a plate tilt of 9 degrees, 350 rpm, and a diameter of 48 cm for the centrifugal rotor. Under these conditions, the volume of the pocket in inner and outer wells differed by 8%, which we found to be an acceptable value.

The obvious question about the possible cross contamination of individual wells during the centrifugation was discussed earlier.[15] The construction of shallow well microtiterplate prevents the transfer of liquid and/or solids from one well to another. The best proof of the absence of cross contamination can be seen in total analyses of all products from each plate (see below).

We have built the dedicated centrifuge with 8 positions for microtiter plates. A computer drives this centrifuge and all centrifugation parameters can be flexibly changed. A 96-channel distributor connected to 6 port selector valve performs the delivery of washing solvents and common reagents. The centrifuge was integrated with the Packard Multiprobe 104 liquid distribution system for the delivery of individual building blocks and reagents. Inclusion of the pipetting system allows us to perform the whole synthesis in a completely automatic regimen. Figure 6 shows the view of this instrument. This compact system can be easily enclosed in inert atmosphere.

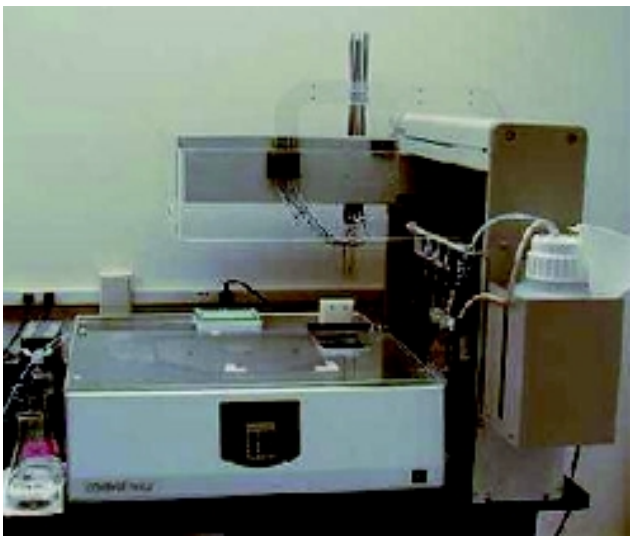

Figure 6 Automated synthesizer combining intelligent centrifuge and pipetting robot Packard Canberra Multiprobe 104

The synthesis starts with distribution of a slurry of solid support into microtiterplates. Plates are then placed onto the perimeter of a rotor with a permanent tilt of 9 degrees. The rotor is rotated at the speed required for complete removal of the liquid portion of the well content. After stopping the rotation, the microtiterplate is placed (rotor is turned) under the multichannel (96 channel) liquid delivery head. The solvent selector valve is turned into the appropriate position and the washing solvent is delivered by actuating the syringe pump. This operation is repeated until all plates are serviced. The rotor is spun at the speed at which the liquid phase is just reaching the edge of the well, thus wetting all solid support in the "pocket", and after reaching this speed, rotation is stopped. The cycle of slow rotation and stopping is repeated, thus, mixing the slurry of solid support in the liquid phase. After shaking for the appropriate time, the plates are spun at high speed. The process of addition and removal of washing solvent is repeated for as many washes are required. The plates are then consecutively placed under the array of 96 openings in the centrifuge cover, and appropriate building block solutions and coupling reagents are delivered by pipetting (Multiprobe 104) through the openings from the stock solutions placed on the centrifuge cover. Alternatively, building blocks are delivered by manual pipetting with a multichannel pipettor from a trough or a prepared "master plate". This alternative is a faster option in the case where the number of building blocks used in the particular step is compatible with logical division of the microtiterplate into rows and columns (4,6,8,12), or when only one building block is distributed over the large part of the plate. When incubation at elevated temperature is required, plates are removed from the centrifuge, stoppered with cap mats and incubated in the shaker oven. After the final washing and drying of the resin in the plate, cleavage can be performed in the same way as described in the "surface suction" section. We have prepared an array of 768 substituted tetrahydroisoquinolinones to demonstrate the simultaneous processing power of the tilted centrifugation technique. The synthetic protocol for preparation of these heterocyclic molecules was developed earlier for synthesis in “tea-bags” and followed the conditions used in the large array synthesis.

Conclusion

Surface suction and tilted centrifugation are very effective and simple methods for liquid removal from a multiplicity of vessels. The surface suction principle is used for the preparation of large libraries in the single compound per well format. The polypropylene microtiterplates were found to be the ideal reaction vessels for tilted centrifugation based synthesis.

Synthesis of an Array of 30,816 Tetrahydroisoquinolinones by Combination of Tea-Bag Technique and Surface Suction in Microtiterplates

Solid support (*p*-methylbenzhydrylamine resin, 1.1 mmol/g, 130 μm, Chem-Impex, Wood Dale, Ill.) was distributed into polypropylene (mesh polypropylene, 100 mesh) bags (1.1 g) and sealed. Resin was swollen in DMF, and 780 bags were shaken for 20 min. in 50% piperidine/DMF to neutralize the resin. The bags were then washed four times with DMF, once with 0.3 M HOBt/DMF, and then four more times with DMF (the last wash contained 0.01% bromophenol blue for monitoring the subsequent acylation step [16]). A solution of bromoacetic acid in DMF (1 M and 8.68 equivalents) was added together with DIC (1.2 M) and the mixture was shaken in polypropylene flask for 1–2 h. After disappearance of the blue coloration, the bags were washed three times with DMF and separated into groups of 30 bags. Each group was washed two times with DMSO and the solution of an amine (1 M) in DMSO was added. The bags were shaken 18–24 h, washed once with DMSO, and then five times with DMF (last wash containing bromophenol blue). A solution of Fmoc-beta-alanine was added (0.3 M and 3 equivalents) together with DIC (0.3 M, 3 equivalents), HOBt (0.3 M, 3 equivalents) and a 1/10 of equivalent of DMAP. After disappearance of the blue coloration in bags not containing tertiary amine groups (these bags remain blue), the bags were washed three times by DMF and four times with CH_2Cl_2. The bags were then laid out in the hood to air dry. When the resin was dry, a small sample of resin from each amine group was taken, cleaved with HF, and analyzed. Only those tea-bags which had the expected material in a purity of 90% or greater were carried through to the next step (Fmoc-deprotection). To remove the Fmoc group from the beta-alanine, a solution of 50% piperidine in DMF was added. After 20 minutes of incubation, the bags were washed with DMF five times and separated into groups of 24 (3 amines in the R1 position did not pass QC, total number of processed bags dropped to 690). The appropriate aldehyde solutions (0.8 M in DMF) were combined with an equal volume of 1.6 M trimethylorthoformate (TMOF) in DMF and then were added to each group of 24 bags. After 3 h of incubation, the liquid was removed and two washes with 0.2 M TMOF/DMF were performed. A solution of homophthalic anhydride (0.4 M in DMF and 5 equivalents) with DIEA (0.03 M) was added to each bottle and bags were shaken overnight. The liquid was removed and six washes with DMF were performed, followed by three washes with *tert*-butyl methyl ether. At this stage the bags were dried again, and a sample from each bag was taken, placed into a well of a microtiterplate and exposed to gaseous HF for 2 h. Samples were extracted and analyzed by LC-MS. Only the bags containing expected product in purity better than 85% (evaporative light scattering detection) were taken to the next step of synthesis (48 bags were removed and 642 bags were processed further). In this step 90% of the content of each bag was divided into 48 wells of a deep well microtiterplate. A set of "master plates" of 48 amine solutions (1 M in DMF) was created in deep well microtiterplates by automatic pipetting from stock solutions (Multiprobe 104, Packard Canberra, Meriden, CT). A solution of HATU (0.3 M in DMF) was added to all wells of each plate by multichannel pipetting (Quadra 96, TomTec) and, after 20 minutes incubation, the appropriate amine solutions were added to each well by pipetting from the "master plates" (the HATU solution remains in the wells during this reaction). After overnight incubation of closed microtiterplates on the shaker, the solution was removed by surface suction and incubation with HATU and amine solution was repeated overnight, once again. The solution was removed by surface suction and microtiterplates were washed with DMF ten times and with 50% *tert*-butyl methyl ether/CH_2Cl_2 four times. The plates were air dried for two days and then placed in a polypropylene chamber. The chamber was flushed with N_2 for 30 min and then filled with gaseous HF. After 2 h at r.t., the chamber was flushed with N_2 overnight, and plates were removed and placed in the desiccator. After overnight evacuation the plates were placed onto the table of the Multiprobe 208 (Packard Canberra, Meriden, CT) and solid support was extracted by repeated (four times) addition and removal of 165 μL of HOAc into the individual wells of microtiterplate. The extracts were transferred to deep well polypropylene microtiterplates and were evaporated in the GeneVac, or lyophilized. All wells were analyzed by flow injection MS and one row of each plate was analyzed by HPLC and ELSD. The results are given in the Table.

Table Purities of Tetrahydroisoqinolinones Synthesized by Combination of Tea-Bag and Surface Suction Techniques

Plate type	Number	Plate MS "hit rate"	HPLC sample purity
QC Passed	263	90.52 %	80.64 %
QC Failed	58	48.63 %	78.49 %
Total	321	82.17 %	80.21 %

Synthesis of an Array of 768 Tetrahydroisoquinolinones by Tilted Centrifugation Technique

The solid support (*p*-methylbenzhydrylamine resin, 1.1 mmol/g, 130 μm, Chem-Impex, Wood Dale, Ill.) was allowed to swell in DMF. The resin slurry was then distributed into the wells of eight polypropylene shallow well microtiterplates (5 mg of resin per well). The microtiterplates were placed on the centrifugal rotor in a tilted position (9-degree tilt) and solvent was removed by centrifugation at 350 rpm. The resin was neutralized with a 5% DIEA/DMF solution, and washed six times with DMF (the last wash contained 0.01% bromophenol blue for monitoring the subsequent acylation step [16]). After six additional DMF washes, a solution of bromoacetic acid (1 M) and DIC (1.2 M) in DMF (100 μL/well) was added, and the mixture was oscillated for 2 h. After the disappearance of blue coloration, the plates were washed six times with DMF, four times with DMSO and a solution of an amine (1 M) in DMSO was added. The plates were shaken overnight, and then washed once with DMSO and five times with DMF (last wash containing bromophenol blue). A solution of Fmoc-beta-alanine in 0.3 M HOBt and DIC was added (100 μL, 0.3 M). After disappearance of the blue coloration in plate wells not containing tertiary amine groups (these wells remain blue), the plates were washed four times with DMF and a solution of 50% piperidine in DMF was added. After 20 minutes of incubation the plates were washed five times by DMF. The aldehyde solutions (0.8 M) were combined with an equal volume of 1.6 M TMOF/DMF and added to the appropriate wells of each plate (100 μL). After a 3 h incubation, the liquid was removed and two washes with 0.2 M TMOF/DMF were performed. A solution of homophthalic anhydride (75 μL, 0.4 M in DMF) with DIEA (0.03 M) was added and plates were shaken overnight. The liquid was removed and six washes with DMF were performed. A solution of HATU (0.3 M in DMF, 75 μL) was added to each well. After 20 min incubation, a solution of an appropriate amine (1 M in DMF, 75 μL) was added. After overnight incubation of closed microtiterplates on the shaker, the solution was removed by centrifugation and the process of preincubation with HATU and incubation with amine solution was repeated and allowed to react overnight once again. The solution was removed by centrifugation and all microtiterplates were washed with DMF twelve times and with *tert*-butyl methyl ether six times. The plates were air dried for three days and placed in a polypropylene chamber. The chamber was flushed with N_2 for 30 min and then filled with gaseous HF. After 2 h at r.t., chamber was flushed with nitrogen overnight and the plates were removed and placed in the desiccator. After overnight evacuation the plates were

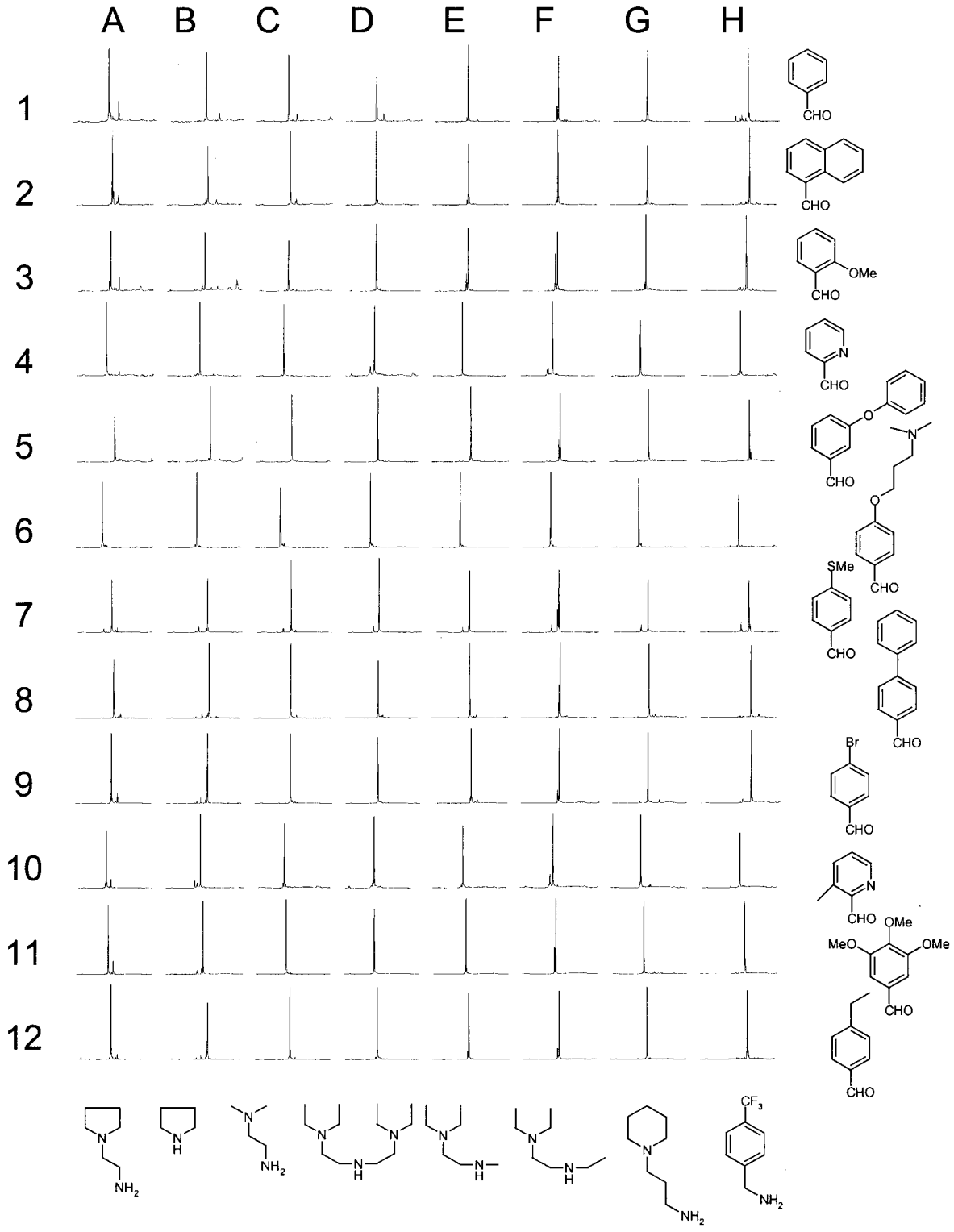

Figure 7 HPLC traces of products synthesized in one microtiterplate

placed onto the table of the Multiprobe 208 (Packard Canberra, Meriden, CT) and the solid support was extracted by repeated (four times) addition and removal of 150 μL of HOAc into the individual wells of the microtiterplate. The extracts were transferred to deep well polypropylene microtiterplates, the content was frozen and HOAc was removed in the lyophilizer. All wells were analyzed by LCMS. The average purity of the prepared compounds was 87%. The HPLC traces of the compounds synthesized in one plate are shown in Figure 7.

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