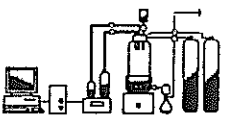
ロボット合成研究会　第３回公開講演会



次世代自動合成のサイエンス&テクノロジー　－講演・展示－

日　時　平成11年７月30日（金）9時30分～17時

会　場　大阪科学技術センター８階中ホール

講　演

Osaka Univ.　Ilhyong Ryu

ryu@chem.eng.osaka-u.ac.jp

付設展示出展企業

（＊はショートプレゼンテーション参加企業：11:35～12:05）

㈱エス・ティ・ジャパン＊、テカンジャパン㈱、東京理化器械㈱＊

日本電子㈱＊、㈱バイオット、㈱モリテックス＊

㈱ユニフレックス＊、㈱ワイエムシイ＊

主　催　近畿化学協会合成部会ロボット合成研究会

協　賛　日本化学近畿支部・有機合成化学協会関西支部

日本薬学会近畿支部・コンビナトリアルケミストリー研究会

近畿化学協会

NEW APPROACHES TO AUTOMATIC SYNTHESIS OF LARGE ARRAYS OF SMALL ORGANIC MOLECULES

Michal Lebl

Spyder Instruments Inc., 9390 Towne Centre Drive, Suite 200, San Diego, CA 92121, U.S.A.

ABSTRACT

Two new methods for the synthesis of large arrays of small organic molecules are described and expemplified on the example of synthesis of libraries 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".

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 works of Leznoff [5], Patchornik [6], Camps [7], and others were 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: 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).

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 welcome 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. 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 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 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

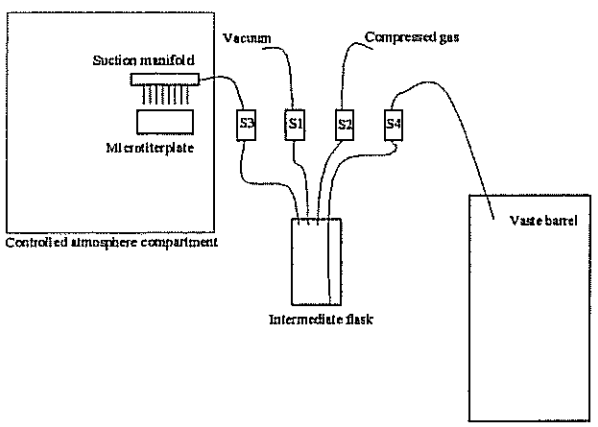


Figure 1. Scheme of liquid transfer conserving inert atmosphere in the robotic envelope.

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[13] 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-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 dessicators 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

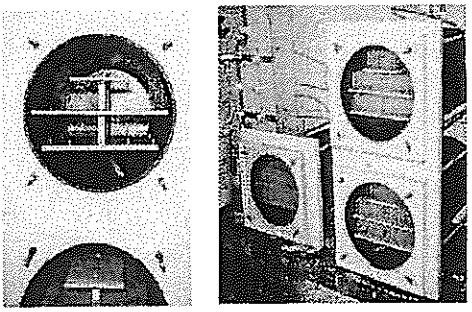


Figure 2. Polypropylene chambers used for cleavage of compounds from benzhydrylamine resin by exposure to gaseous HF. The window is made of polymethacrylate covered with polypropylene foil.

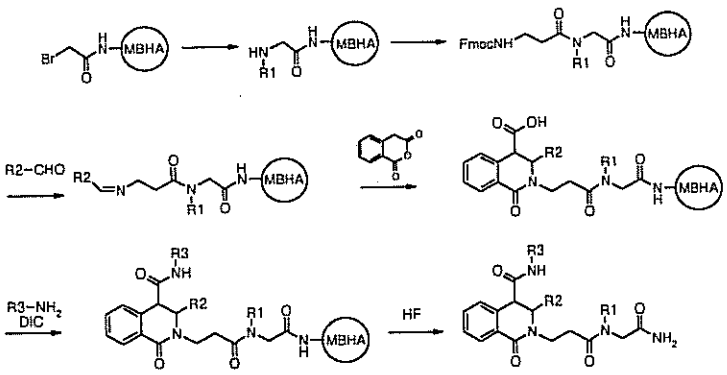


Figure 3. Synthetic scheme for library of tetrahydroisoquinolinones.

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. We have 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 [14] was modified for the "surface suction" technique. The purities of the prepared compounds are given in Table 1. 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 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.

Table 1. 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 %

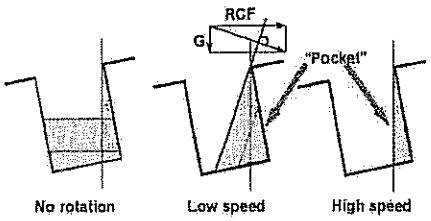


Figure 4. Principle of "tilted centrifugation".

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"[15]. The principle of tilted centrifugation is shown in Figure 4. 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 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

Table 2. Volumes of "pockets" in wells A and B (see figure 4) under different conditions of centrifugation.

Centrifuge parameters				RCFs (x G)		Volumes of wells (µl)			Ratio of
Tilt	Radius of		Speed	Inner	Outer	At infinity	Inner	Outer	volumes
(deg)	Inner well A (cm)	Outer well B (cm)	(rpm)	well A	well B	RCF	well A	well B	A/B
9	17.4	24	200	12.5	17.3	29.6	68	56.6	1.2
9	17.4	24	350	38.4	52.9	29.6	41.2	37.9	1.09
9	17.4	24	500	78.3	108	29.6	35.2	33.6	1.05
9	17.4	24	1000	313.2	432	29.6	31	30.6	1.01
1	17.4	24	350	38.4	52.9	1.2	7	5.1	1.37
3	17.4	24	350	38.4	52.9	6	13.9	11.5	1.21
5	17.4	24	350	38.4	52.9	12.6	22.1	19.3	1.15
15	17.4	24	350	38.4	52.9	61.1	74.7	70.9	1.05
9	7.3	14.2	200	5.3	10.2	29.6	126.7	77.5	1.63
9	7.3	14.2	350	16.1	31.3	29.6	58.8	44	1.34
9	7.3	14.2	500	32.9	63.9	29.6	43.3	36.5	1.19
9	7.3	14.2	1000	131.4	255.6	29.6	32.9	31.3	1.05
1	7.3	14.2	350	16.1	31.3	1.2	18.9	8.7	2.17
3	7.3	14.2	350	16.1	31.3	6	27.7	16	1.73
5	7.3	14.2	350	16.1	31.3	12.6	37.4	24.4	1.53
15	7.3	14.2	350	16.1	31.3	61.1	94.2	77.8	1.21

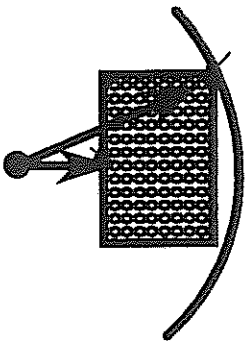


Figure 5. Situation of wells in microtiterplates placed on the perimeter of the centrifuge.

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. Figure 5 illustrates this situation and Table 2 gives the data calculated for the wells A (the closest to the center of rotation) and B (the most distant one). 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 of the plate. Plates placed on a rotor of very large diameter, or on a rotor which is spinning very fast, will have an insignificant difference between the forces exerted onto "inside" and "outside" wells. 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.

If the wells were created by drilling into an inert material, the liquid expelled from one well would inadvertently enter another well placed closer to the perimeter of the centrifuge. However, a 96 well shallow microtiterplate is actually composed of 96 small cylinders attached to a flat polypropylene sheet and connected by a thin "rib", thus creating an array of 96 round wells plus 117 interwell spaces. The liquid expelled by centrifugal force from each well comes into the interwell space, flies across this space, and ends up on the outer wall of the adjacent well (see Figure 6). It then flows along the well until it detaches and flies across another interwell space, eventually ending at the edge of the plate from where it flies onto the wall of the centrifuge drum. We have tested the transfer of liquid and/or solid material from one well into another in several ways. We have loaded the wells with an amount of colorized solid support (resin) which exceeded the capacity of the pocket and observed the fate of the resin expelled from the well. Overflow of the resin ended in the interwell space and we have not observed any transfer of the resin beads into adjacent wells. In another experiment, we analyzed products synthesized in all wells of the microtiterplate by HPLC and mass spectroscopy. We have not found any traces of contamination by liquid or solid transfer between wells in our model experiments.

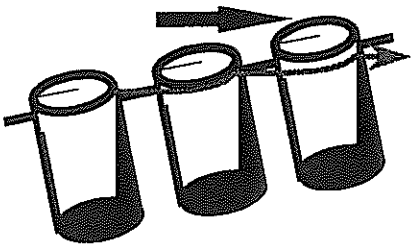


Figure 6. Trajectory of liquid expelled from the microtiterplate well.

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 7 shows the view of this instrument. This compact system can be easily enclosed in inert atmosphere.

The synthesis is performed in the following way. A microtiterplate with a slurry of solid support distributed into it is placed on 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

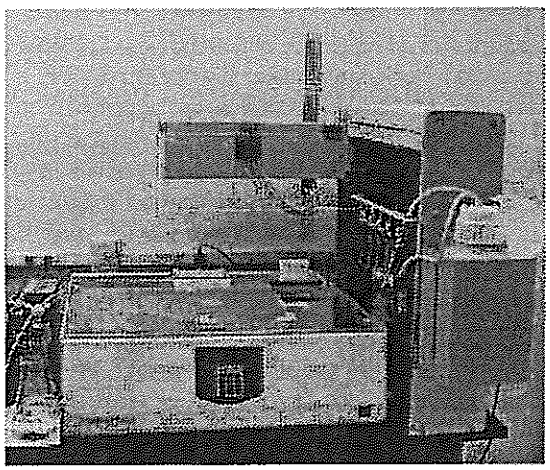
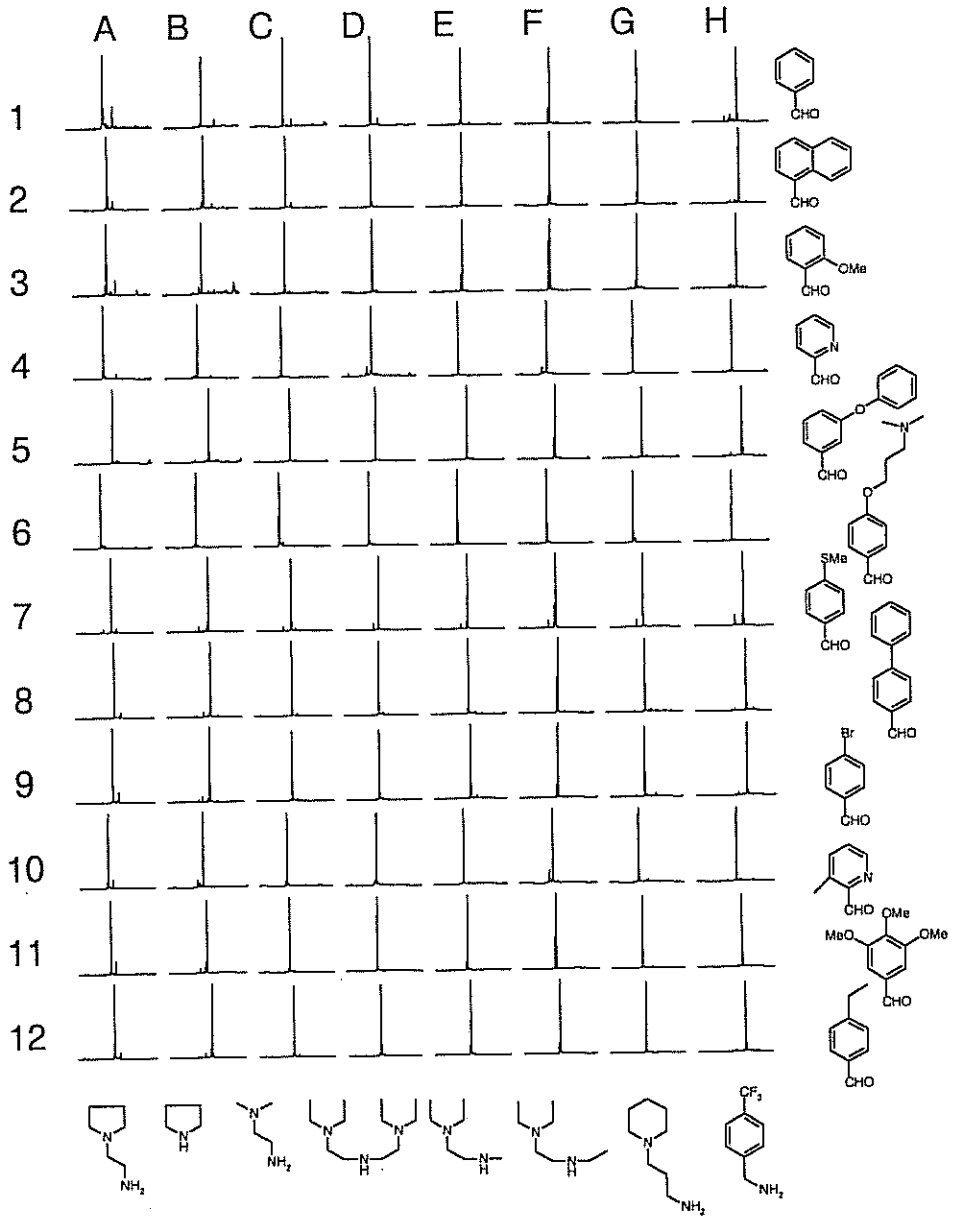


Figure 7. Automated synthesizer combining intelligent centrifuge and pipetting robot Packard Canberra Multiprobe 104.



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 the elevated temperature is required, plates are removed from the centrifuge, stoppered with the cap mats and incubated in the shaker oven. After the final wash 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. See HPLC traces of compounds from one plate.

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. The fact that tilted centrifugation is the only way for removal of liquids from an unlimited number of reaction vessels simultaneously is suggesting its application in ultraminiaturized synthesizers.

ACKNOWLEDGEMENT

Part of this work (tilted centrifugation technique development) was supported by SBIR NIH grant IR43GM58981-01 to Spyder Instruments, Inc. Described experiments were performed at Trega Biosciences, Inc.

References:

1. Leblova Z., Lebl M. Compilation of papers in molecular diversity field. 1999. INTERNET World Wide Web address: http://www.5z.com.
2. Merrifield R. B. *J.Amer.Chem.Soc.* **1963,** *85,* 2149.
3. Letsinger R. L.; Kornet M. J. *J.Amer.Chem.Soc.* **1963,** *85,* 3045.
4. Bunin B. A.; Ellman J. A. *J.Amer.Chem.Soc.* **1992,** *114,* 10997.
5. Leznoff C. C.; Wong J. Y. *Can.J.Chem.* **1972,** *50,* 2892.
6. Patchornik A.; Kraus M. A. *J.Amer.Chem.Soc.* **1970,** *92,* 7587.
7. Camps F.; Castells J.; Font J.; Vela F. *Tetrahedron Lett.* **1971,** 1715.
8. Cargill J. F.; Lebl M. *Curr.Opin.Chem.Biol.* **1997,** *1,* 67.
9. Lebl M.; Krchnák V. Techniques for massively parallel synthesis of small organic molecules. In *Innovation and Perspectives in Solid Phase Synthesis & Combinatorial Libraries,* Epton R., Ed.; Mayflower Scientific Limited: Birmingham, 1998;
10. Krchnák V.; Weichsel A. S.; Lebl M.; Felder S. *Bioorg.Med.Chem.Lett.* **1997,** *7,* 1013.
11. Houghten R. A. *Proc.Natl.Acad.Sci.USA* **1985,** *82,* 5131.
12. Griffith M. C.; Dooley C. T.; Houghten R. A.; Kiely J. S. In *Molecular Diversity and Combinatorial Chemistry. Libraries and Drug Discovery,* Chaiken I. M., Janda K. D., Eds.; American Chemical Society: Washington,DC, 1996; p. 50.
13. Lebl M. *Bioorg. Med. Chem. Lett.* **1999,** *9,* 1305; *J.Assoc.Lab.Autom.* **1998,** *3,* 59.