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Evaluation of Gaseous Hydrogen Fluoride as a Convenient Reagent for Parallel Cleavage from the Solid Support

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We have tested the limits of gaseous hydrogen fluoride as an agent for parallel detachment of organic molecules from the solid support. Peptides were chosen as relatively sensitive models for this reaction. Acid-catalyzed amide bond hydrolysis, side chain modification (tryptophan and other unnatural amino acids) by the protecting group residues as well as dehydration of serine and asparagine was followed. The technique of cleavage of side chain protection prior to the resin cleavage has given satisfactory results. Two-step deprotection and cleavage from benzhydrylamine resin by TFA and HF was compared to the deprotection and cleavage by TFA from Knorr resin.

Demands of high throughput screening are being met by synthesis of mega-arrays of organic molecules. The most common method of preparation of these large collections of compounds is the parallel solid phase synthesis. The technique of "bag explosion" in which intermediates are prepared in hundreds of "tea-bags" and then distributed into individual wells of hundreds of microtiter plates processed by robotic stations is practiced routinely by Trega Biosciences for preparation of large libraries (10−60000 members).[1,2] The technique of parallel synthesis of several hundred compounds by "tilted centrifugation" is being commercialized by Spyder Instruments.[3] The last step in both of these techniques is cleavage of the compound from the solid support in several hundred or tens of thousands of wells distributed in up to several hundred of microtiter plates. The most convenient way of detachment of the product from the solid support is the application of a gaseous reagent, which can be simultaneously applied to (and removed from) a basically unlimited number of synthetic compartments—in our case wells of multiple microtiter plates.

Gaseous reagents were used in several laboratories for cleaving products from solid support. Gaseous ammonia was recognized as the reagent of choice for simultaneous cleavage of peptide arrays from Geysen's pins.[4,5] It was applied for detachment of peptides from Merrifield-type polystyrene supports in the industrial scale (several kilogram batches of resin).[6] Ammonia was studied carefully as a reagent for cleavage from cotton.[7] Gaseous ammonia was also used for cleavage of oligonucleotides from CPG support.[8] Trifluoroacetic acid vapors were used for cleavage from acid-labile (benzhydrylamine[9,10] or trityl[11]) linkers.

Even though liquid hydrogen fluoride is used routinely in the solid phase synthesis of peptides,[12−15] its application in gaseous form is relatively recent. We have constructed a battery of polypropylene "drums" which can house up to 54 deep-well microtiter plates for cleaving compounds derived from the production of "bag explosion" synthesis[1,2] and a small "bazooka" which is designed for the cleavage of nine shallow-well microtiter plates with products from the "tilted centrifugation" process. Gaseous HF was proven as the method of choice for cleavage of organic compounds from the benzhydrylamine resin, and several hundred thousand compounds were prepared this way at Trega.[2] However, the building blocks used for the synthesis of these compounds were selected by application of the whole process, and only those providing clean products were used. We were interested to find the limitations of this method of cleavage. Here we show the results from the cleavage of arrays of peptides with natural and unnatural residues prepared as models for demonstration of the use of gaseous HF in cleavage of these relatively sensitive compounds.

There are several dangers in the application of strong acid in the deprotection and cleavage of peptide from the solid support. Acidolytic cleavage proceeding by the SN_1 mechanism generates cationic species, which can alkylate the sensitive side chains (Tyr, Trp, Cys, Met), the result being a modified peptide or lowered yield due to reattachment of the peptide to the resin. Cleavage in the presence of water results in significant amide bond hydrolysis. In the standard procedure, HF is applied as a liquid mixture with various scavengers, preventing damage to the peptide by capturing the reactive species. The optimized method for the HF

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[§] Liquid applied to the wells of microtiter plate may result in uneven condensation (dissolution) of HF in individual wells. Very instructive was an experiment in which 20 μL of DMF was placed in one well of an empty deep-well (2 mL volume) microtiter plate and the plate was placed in the HF chamber. In several minutes after introduction of HF gas, the well was overflowing with a solution of 99% HF in DMF. Obviously, the resin going for the HF gas treatment must be free of components (such as DMF), which would promote uncontrollable HF condensation. Very often DMF is trapped in the resin, and its removal is difficult. Very thorough washing and drying before the cleavage step is extremely important.

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Table 1. Protocol for Solid Phase Synthesis

addition of DMF (150 μL) and centrifugation	5 times
addition of 2% HOBt/DMF (150 μL) and centrifugation	2 times
addition of 2% HOBt and 0.005% bromophenol blue in DMF (150 μL)	
centrifugation	
addition of 0.3 M Fmoc-amino acid in 0.3 M HOBt and 0.3 M DIC in DMF (100 μL)	
shaking until resin is not blue (2−12 h)	
centrifugation	
addition of DMF (150 μL) and centrifugation	5 times
addition of 50% piperidine/DMF (150 μL)	
shaking 2 min	
centrifugation	
addition of 50% piperidine/DMF (150 μL)	
shaking 10 min	
centrifugation	

cleavage utilizes a two-step process in which the first step ("low HF") uses the mixture of HF in dimethyl sulfide and *p*-cresol (25:65:10) and in the second step ("high HF") the dimethyl sulfide is replaced by HF (90:10). Since it is inconvenient (or impossible) to apply scavengers in the gaseous HF cleavage procedure,[§] we have tested the cleavage without scavengers and with scavengers applied by "soaking" the resin in vapors of toluene and/or anisole.

Application of linkers that are cleavable by trifluoroacetic acid is relatively common in the Fmoc method of peptide synthesis. However, this method requires the cleavage and deprotection of side chains in one step. This results in the generation of relatively long-lived cations from protecting groups which have to be scavenged by various additives. Due to the fact that in multiple synthesis performed in the microtiter plates the simple purification by precipitation is rather complicated, the only way for removal of the scavengers is evaporation, which limits the selection of possibilities. On the other hand, synthesis by Fmoc method on the relatively acid stable benzhydrylamine type resin allows deprotection of the side chains by pretreatment with trifluoroacetic acid and thorough washing before the application of gaseous HF. HF is therefore applied only for the detachment from the resin, and the only side reaction (involving other reagents than peptide itself and HF) is reattachment of the product to the resin. This reaction can decrease the yield of the product, but it should not have any influence on the purity of the synthesized compound. We have compared the synthesis of the same set of peptides on *p*-methylbenzhydrylamine resin and on the Knorr linker.

Experimental Section

Peptide Synthesis. The resin (*p*-methylbenzhydrylamine (MBHA) resin, 1.2 mmol/g, ChemImpex, Woods Dale, IL, or aminomethylpolystyrene, 0.7 mmol/g, onto which Knorr linker (Advanced ChemTech, Louisville, KY) was attached) was distributed (5 mg/well) into individual wells of shallow-well polypropylene microtiter plate (Evergreen Scientific, Los Angeles, CA) by multichannel pipetting from a polypropylene trough. The plates were placed in the rotor of Compas 768 (Spyder Instruments, San Diego, CA) under a permanent 9° tilt inward and were washed by repeated addition of appropriate solvents and centrifugation. Fmoc protected α-amino acids were from Advanced ChemTech (Louisville, KY). β-Amino acids were from PepTech (Cambridge, MA) and CSPS (San Diego, CA). The protocol for the synthesis is given in Table 1.

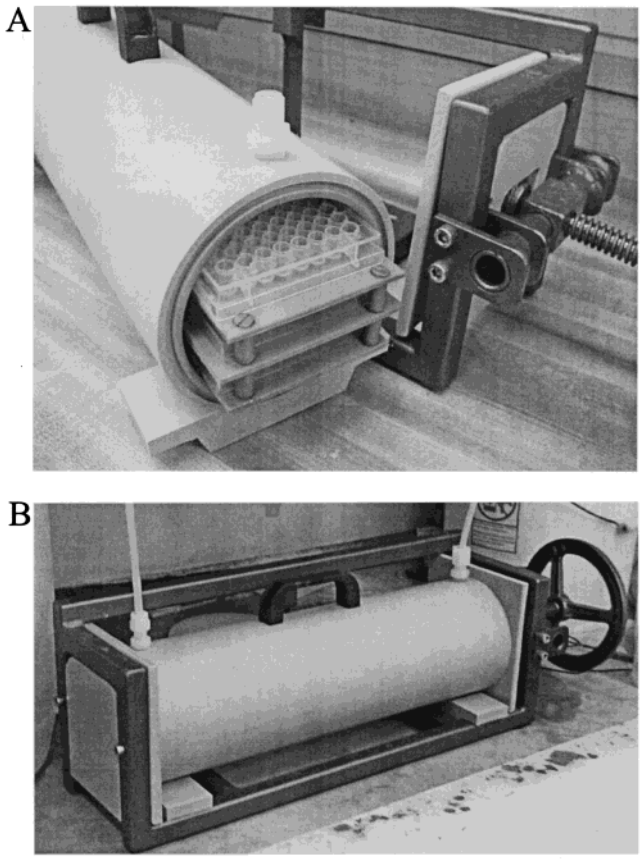

Figure 1. Polypropylene HF chamber ("bazooka") for simultaneous cleavage of up to nine shallow-well polypropylene microtiter plates. A—open chamber; B—chamber attached to HF apparatus.

Deprotection and Cleavage. (1) MBHA Resin. The plates were washed with DMF (5 times) and methanol (5 times) and allowed to air-dry. A TFA/anisole/DCM mixture (50:5:45) was added by multichannel pipetting (150 μL per well). After 10 min the plates were placed into a SpeedVac (Savant Instruments, Holbrook, NY), and TFA was evaporated in vacuo. The plates were placed into the Compas 768 and washed by DMF (10 times) and MeOH (5 times). After drying in a SpeedVac overnight, plates were placed in the polypropylene chamber (available from Spyder Instruments) (see Figure 1) together with a plate containing 15 mL of anisole (open area 100 cm^2), and the chamber was closed for 5 h.[‖] The anisole container was removed, and the chamber was then attached to an HF apparatus and flushed by nitrogen

[‖] In an independent experiment the amount of anisole which will be absorbed by the dry polystyrene resin was determined—300 mg of resin absorbs 28−39 mg of anisole in 12 h (empty plate absorbs about 3 mg). This amount is roughly equimolar to the amount of the compound synthesized on the resin.

[⊥] Extreme caution must be exercised when working with HF as it is extremely toxic and causes dangerous burns. Study of the appropriate literature before using HF is required. However, all operations are performed in a completely enclosed apparatus connected to the atmosphere through the series of water absorbers and the chamber is opened only after HF was replaced by nitrogen, and therefore it can be considered rather safe.

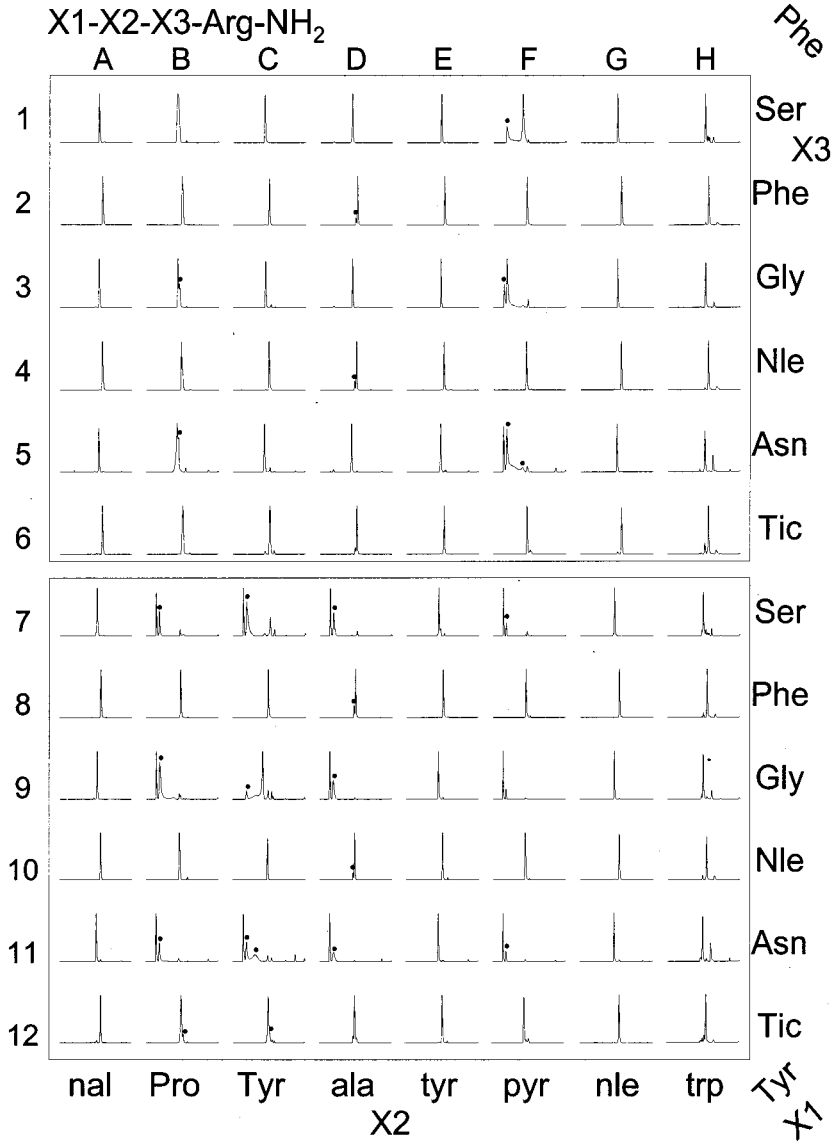

Figure 2. HPLC traces of all peptides synthesized in one microtiter plate. *X*-scale of each trace—0 to 6 min; *Y*-scale—total ion current. Gradient 5−95% acetonitrile in 0.05% TFA/water in 5.5 min, column Keystone C-18 (4.6 × 50 mm), flow 1.5 mL/min, Hewlett-Packard HP 1050, mass spectrometer Finnigan Mat LCQ operating in electrospray positive mode. In all cases the major peak contained the expected product. Dots next to the smaller peaks denote that the peak contains product with expected molecular weight.

for 20 min.[⊥] HF was introduced from the cylinder heated in a water bath to 40 °C. After 15 min of HF flow (bubbles in the water trap decreased significantly in size as the nitrogen was replaced by HF which quickly dissolves in water), the chamber was isolated and left closed for 90 min. The chamber was flushed by nitrogen for 2 h, and plates were transferred into the desiccator and evacuated over NaOH pellets overnight.[▽] The product was extracted by repeated introduction of 150 μL of acetic acid by the automatic pipettor Multiprobe 104 (Packard Canberra, Meriden, CT). Samples for HPLC/MS analysis were taken, and the solution was evaporated in SpeedVac.

(2) Knorr Linker Resin. The plates were dried, and TFA/anisole/water mixture (92:3:5) was added by multichannel pipetting (200 μL per well). After 2 h the plates were placed into SpeedVac, and TFA was evaporated in vacuo. The product was extracted and analyzed as above.

[▽] Plate removed from the chamber is usually bright orange (plate which was not pretreated with anisole is brown-orange) and after evacuation loses most of the coloration.

Discussion

Time demands limit the complete analysis of compounds from production of Compas 768. If one analysis would take only 20 min, we would be able to analyze only 72 compounds per day. In the routine production we completely analyze only a sample (10−20%) of all compounds by LC/MS. However, all compounds are subjected to flow injection MS analysis. For the presented study, however, we ran all compounds through LC/MS.

The results of LC/MS analyses were graphically presented in a 96-well plate-like format. We found this presentation of the data very instructive since it shows clearly the influences of and dependences between individual building blocks (amino acids). Figure 2 shows the LC/MS analyses of all wells of one plate from the synthesis of a library of 768 individual tetrapeptides. The library was composed of

Table 2. Composition of the Tetrapeptide Library of 768 Compounds Prepared on Benzhydrylamine Resin

position 1	position 2	position 3	position 4
D-2-naphthylalanine (nal)	**D-2-naphthylalanine (nal)**	**L-serine (Ser)**	**L-arginine (Arg)**
L-tryptophan (Trp)	**L-proline (Pro)**	**L-phenylalanine (Phe)**	L-2-naphthylalanine (Nal)
L-phenylalanine (Phe)	**L-tyrosine (Tyr)**	**glycine (Gly)**	
L-tyrosine (Tyr)	**D-alanine (ala)**	**L-norleucine (Nle)**	
L-alanine (Ala)	**D-tyrosine (tyr)**	**L-asparagine (Asn)**	
D-tyrosine (tyr)	**D-3-pyridylalanine (pyr)**	**L-tetrahydroisoquinoline-3-carboxylic acid (Tic)**	
L-2-naphthylalanine (Nal)	**D-norleucine (nle)**		
(4-aminomethyl)benzoic acid (paba)	**D-tryptophan (trp)**		

Table 3. Composition of a Library of 384 Analogues of D-Tyr-D-Nle-Gly-L-Nal, D-Phe-D-Phe-D-Ile-D-Arg, and L-Trp-D-Tyr-L-Nva-L-Arg[a]

position 1	position 2	position 3	position 4
D-phenylalanine (phe)	D-phenylalanine (phe)	D-isoleucine (ile)	L-arginine (Arg)
L-tyrosine (Tyr)	D-norleucine (Nle)	glycine (Gly)	D-arginine (arg)
L-arginine (Arg)	(*S*)-3-amino-4-(4-methylphenyl)-butyric acid (Bmp)	L-norvaline (Nva)	L-2-naphthylalanine (Nal)
L-tryptophan (Trp)	(*S*)-3-amino-5-phenyl-pentanoic acid (Bhp)	L-tyrosine (Tyr)	(*S*)-3-amino-4-(2-naphthyl)-butyric acid (B2n)
(*S*)-3-amino-4-(4-methylphenyl)-butyric acid (Bmp)	(*R*)-3-amino-3-phenyl-propionic acid (bpg)	(*S*)-3-amino-4-(3-cyanophenyl)-butyric acid (B3c)	(*S*)-3-amino-4-(1-naphthyl)-butyric acid (B1n)
(*S*)-3-amino-5-phenyl-pentanoic acid (Bhp)	(*S*)-3-amino-4-(3-cyanophenyl)-butyric acid (B3c)	(*S*)-3-amino-4-(4-cyanophenyl)-butyric acid (B4c)	
(*R*)-3-amino-3-phenyl-propionic acid (bpg)	(*S*)-3-amino-4-(4-cyanophenyl)-butyric acid (B4c)	(*S*)-3-amino-4-(4-fluorophenyl)-butyric acid (BFp)	
(*S*)-3-amino-4-(3-benzothienyl)-butyric acid (Bbt)	(*S*)-3-amino-4-(4-fluorophenyl)-butyric acid (BFp)		
(*S*)-3-amino-4-(2-thienyl)-butyric acid (Bta)			

[a] Not all possible 2520 combinations were prepared.

all possible combinations of peptides with two amino acids in position 4, six in position 3, and eight in positions 1 and 2 (for composition of the library, see Table 2). Amino acids printed in bold in Table 2 composed peptides in Figure 2. In all cases, the major peak contained compound with expected molecular weight. The peaks marked with the dot contained a product with molecular weight identical to that contained in the major peak. Explanation for these peaks can be found in existence of cis and trans isomers, which can be expected in peptides containing proline (see column B, rows 3, 4, and 5; also note peak broadening in rows 1, 2, 6, and 10). Peaks with shortest retention time in wells B7, B9, B11, C7, C11, D7, D9, D11, F3, F5, F7, F9, and F11 are caused by compounds "breaking through" the column (eluted in the dead volume of the column) due to the too large volume of injected sample and too fast gradient. Analysis of these compounds under standard HPLC conditions confirmed this assumption. The pattern of the elution in C7, C9, C11, F1, F3, and F5 is typical for compounds eluting as equilibrium of conformers.[16]

The real impurities we have observed are in peptides containing tryptophan (column H), serine (rows 1 and 7), asparagine (rows 5 and 11), and 3-pyridylalanine (column F). The extent of the Pmc (2,2,5,7,8-pentamethylchroman-6-sulfonyl) arginine side chain protecting group transfer onto the tryptophan residue depends strongly on the peptide sequence. When Arg and Trp residues are separated by hydrophobic residues, the alkylation is much less pronounced than in the case of hydrophilic residues. However, the extent of this reaction is clearly acceptable, considering the conditions of deprotection (anisole and water as scavengers, evaporation to dryness) and cleavage (HF gas at room temperature). Serine and asparagine side chains did not experience any significant dehydration, and we did not observe any peptide bond hydrolysis.

In another example we have prepared analogues of three biologically active tetrapeptides found in combinatorial libraries as ligands for μ-, κ-, and δ-opioid receptors (D-Tyr-D-Nle-Gly-L-Nal, D-Phe-D-Phe-D-Ile-D-Arg, and L-Trp-D-Tyr-L-Nva-L-Arg). For the synthesis of 384 analogues, the set of optically active β-amino acids was used. The analogues were composed from amino acids listed in Table 3. (Results of biological evaluation of these analogues will be presented elsewhere.) The peptides in Figure 3 were synthesized on MBHA resin and cleaved by gaseous HF. The same peptides were synthesized in parallel on Knorr linker and cleaved by TFA. Results are shown in the same figure as traces in rows marked b. As you can see, the results from MBHA resin are substantially better, confirming that the removal of the side chain protecting groups prior to the cleavage provides cleaner products. The most significant impurities in the case of peptides prepared on Knorr linker were peptides with incompletely deprotected arginine residue. (Prolonged treatment with TFA decreases the content of this impurity.) Most of the peptides were obtained in purity better than 90% (the HPLC traces similar to the rows 1a and 5a in Figure 3). Again, we expected problems in compounds containing tryptophan and arginine separated by two amino acid residues—in this case the transfer of protecting group (Pmc) from the arginine side chain to the tryptophan indole ring is the most pronounced and cannot be prevented by any scavenger mixtures.[17] We observed this transfer in all

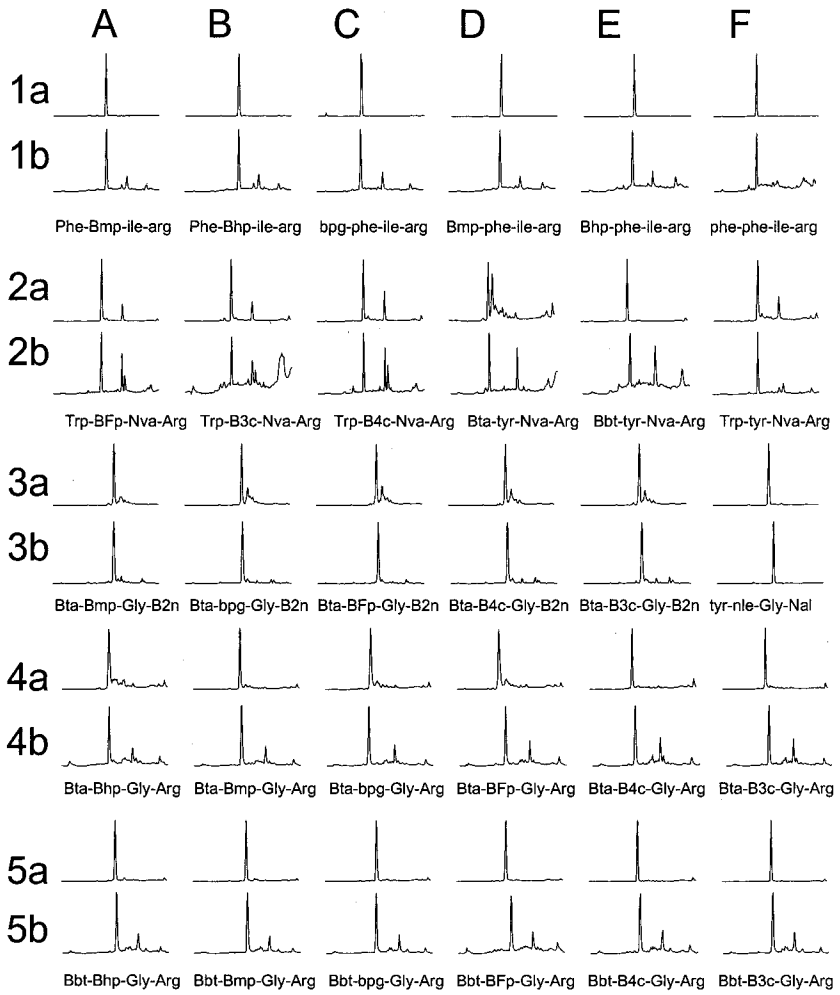

Figure 3. HPLC traces of peptides containing unnatural β-amino acids. Conditions same as in Figure 2. Rows a—peptides cleaved by gaseous hydrogen fluoride from MBHA resin; rows b—peptides cleaved by trifluoroacetic acid from Knorr linker.

peptides containing the Trp-xxx-xxx-Arg sequence (row 2 in Figure 3), but in most of the cases the content of correct peptide was 60−75%. The use of sensitive β-amino acid, Bta (3-amino-4-(2-thienyl)-butyric acid), resulted always in a significant number of impurities in the case of use of gaseous HF (compare traces 3a and 3b). However, some combinations of amino acids in the sequence promoted the difficulties with this amino acid. Surprisingly, the presence of Arg(Pmc) did not result in substantially higher amount of impurities. Compare row 3a and row 4a. Another potentially troublesome β-amino acid, Bbt (3-amino-4-(3-benzothienyl)-butyric acid), produced clean products (row 5a).

Conclusion

Gaseous HF cleavage can be applied to a wide array of products prepared by solid phase synthesis. Even the sensitive sequences of peptides can be processed in this way. The method is convenient for preparation of large arrays of individual compounds. The advantageous feature of synthesis on relatively stable linker is the possibility of removal of all protecting groups and thorough washing before the final cleavage, which can be performed with minimal amounts of scavengers.

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