Solid Phase Chemistry: Direct Monitoring by Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry. A Tool for Combinatorial Chemistry

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The rapid development of combinatorial chemistry[1] has remarkably increased the complexities of the reactions carried out on the solid phase. This is clearly illustrated by the wide spread use of allyl chemistry in peptide cyclizations[2,3] the recent reports of aldol,[4,5] Suzuki,[5] and Diels–Alder[6] reactions on the solid phase as well as the ever increasing use of the solid phase for the synthesis of peptide mimetics, e.g., peptoids.[7] This has brought with it a need to provide a simple and efficient means of monitoring and analyzing such reactions at the molecular level. One such approach has been the highly successfully analysis of derivatized beads by both proton[9] and carbon-13 NMR.[10] Another ideal time and material efficient means of following solid phase reactions would be the direct mass spectrometric analysis from the solid support. Mass spectrometry, a considerably more sensitive analytical tool than other conventional techniques, such as NMR and IR, would provide us with the power to obtain the required information from just a single resin bead.[7,8]

Here we describe the direct analysis by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) of the reaction products from both single beads and small numbers of beads (10–100).[11] This allowed a series of solid phase reactions to be directly monitored in a manner not previously possible. Direct monitoring on such a small number of beads allowed major modifications of reaction protocols in the light of MALDI-TOF MS analysis which clearly showed, in certain cases, that reactions were failing or side reactions were dominating the reaction process. This is clearly an important factor when considering the application of new reactions to the solid phase, when such problems would only come to light following the completed synthesis, compound removal from the resin, and analysis. The simple protocol we report should allow any series of solid

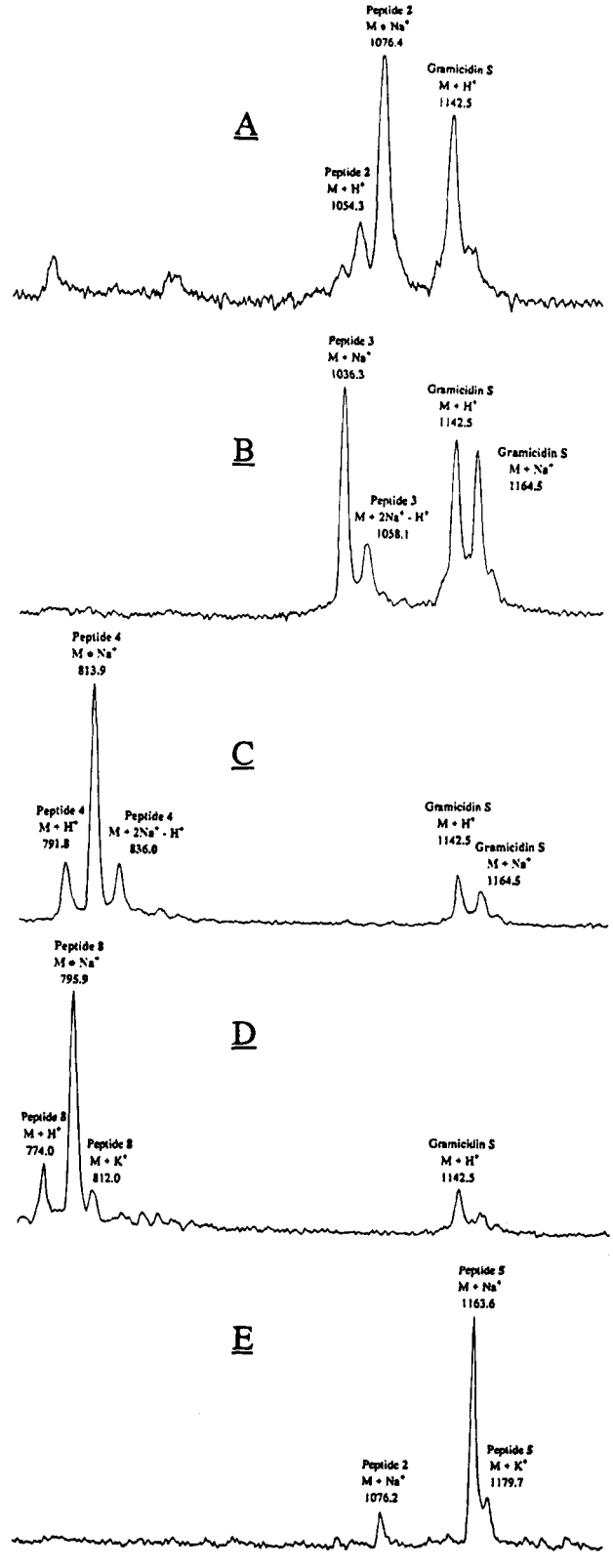

Figure 1. MALDI-TOF mass spectra obtained directly from the resin of the following solid phase reactions: (A) peptide **2**; (B) allyl deprotection of peptide **2**; (C) Fmoc deprotection of peptide **3**; (D) cyclization of peptide **4**; (E) coupling to the carboxyl terminus of peptide **3**. Calibration error; +/− 0.6 Da. Peptides 1–8 defined in ref 20.

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(11) MALDI-TOF MS is a relatively new technique which has caused a renaissance in linear-laser desorption time of flight mass spectrometry. Matrix assistance was developed in 1987[17] for the analysis of large and involatile molecules at low concentrations but has also shown its use in the analysis of peptides in the picomole to femtamole region.[18,19] This means that the process is sensitive enough for the analysis of the reaction products from a single resin bead which typically holds 400 pmol of compound (if 1 g of resin consists of approximately 1×10^6 beads with a substitution of at least 0.4 mmol/g).

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phase organic reactions, which are compatible with the acid lability of the Rink amide linker,[12] to be directly monitored and accurately analyzed within 1 h. To test the strategy, we report the synthesis of **5** and **8** from **1** (peptides **1**–**8** defined in ref 20). This involved monitoring the addition of an amino acid residue to the C-terminus of the peptide a head to tail amide cyclization on the resin as well as the removal of both Fmoc and allyl ester (i.e. prop-2,3-ene ester) protecting groups.

Resin **1** was prepared according to usual techniques[13] and gave a strong sodiated pseudomolecular ion (MNa^+, 1076.4) for the deprotected peptide **2** when directly analyzed from the bead by MALDI-TOF MS[14] (see Figure 1). Three matrices were utilized in the initial studies: 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHC), and 3,5-dimethoxy-4-hydroxycinnamic acid (DHC).[15] Calibration of each spectrum was ideally provided by Na^+/K^+ and gramicidin S (MH^+, 1142.5) as internal calibrants.

A sample of the resin **1** (50 mg) was treated with $Pd(PPh_3)_4$ (0.2 equiv) according to literature procedures.[3,16] The first method utilized[16] proved very unreliable with only very low levels of allyl deprotection being observed by MS analysis. Utilization of a second literature procedure[3] provided a much more reliable method of allyl deprotection to give **3** (MNa^+, 1036.3). However the use of the Pd chelating agent (0.5% DIEA and 0.5% sodium diethyldithiocarbamate in DMF)[3] had to be avoided to prevent concurrent removal of the Fmoc group (10–60% removed) as shown by MS analysis.

Attempted cyclization of peptide **4** on the resin gave a number of interesting side reactions: The use of excess DIC/HOBT showed very clearly, by MS analysis, what is presumably the formation of the *N*-acylurea peptide **6** (MH^+, 917.5), while use of PyBOP/DIEA initially gave high yields of the piperidyl amide **7** (MH^+, 859.0). The latter side reaction was eliminated by washing the allyl and Fmoc deblocked peptide resin with 0.4% concentrated HCl in DMF to remove resin-bound piperidine.[2] Coupling with PyBOP/HOBt/DIEA[2] gave the head to tail cyclic product **8** in high yield (MNa^+, 795.9), i.e. no signals corresponding to **4**, **6**, or **7** being observed (see Figure 1). The ability of the MS approach to detect and allow these various synthetic problems to be rectified clearly demonstrates its utility.

Addition of the amino acid H-Ser(O^{t}Bu)OAllyl to the allyl-deblocked carboxy terminus (activated by PyBOP/HOBt/DIEA) of **3** cleanly gave the elongated peptide **5** (MNa^+, 1163.6) as determined by MALDI-TOF MS (see Figure 1).

In conclusion, we have developed a very simple, fast, and direct method for MALDI-TOF MS analysis of reactions carried out on the solid phase. The protocol utilises only tiny amounts of resin (less than 100 beads or even a single bead) and should allow new solid phase reactions to be tuned and modified quickly and efficiently as long as the chemistry carried out is compatible with the Rink linker. It is probable that the use of other linkers, such as a photolabile linker cleavable by the laser used for MALDI-TOF MS, will extend the utility of this technique.

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(13) Beads utilized were polystyrene based, derivatised with the acid labile "Rink amide linker" with a substitution of approximately 0.4 mmol g^{-1}. Peptide synthesis was carried out using the Fmoc DIC/HOBt strategy on a NovaSyn-Gem peptide synthesizer utilizing counterion distribution monitoring (CDM).

(14) A single bead (removed using microtweezers and a microscope) or beads (approximately 10–100 removed by pipetting from a suspension in CH_2Cl_2) were placed into a well of the sample plate. The stainless steel sample tray was placed onto a metal rack in a glass dish which contained 10 mL of slowly stirred TFA. The glass dish was sealed with a plate of glass, and the sample plate was left in the TFA vapor for typically 30 min at room temperature. Cleavage of the peptide causes a red coloration of the bead(s) due to cation formation, the intensity of which could be used to determine if a longer TFA treatment was necessary. At this time the sample plate was removed from the glass dish, and the matrix solution and internal standards were added to each well (2 μL of either a saturated solutions of CHC or DHC or DHB (100 mg/mL) in water/acetonitrile/TFA (70/30/0.1) plus 1 μL of a solution of gramicidin S (10 μg/mL in water/TFA (100/0.1)). These were left to co-crystallize around the bead(s) for 15–30 min at rt before the plate was loaded into a GSG fOCUS Benchtop II linear-laser desorption time of flight mass spectrometer and the sample irradiated with a 337 nm laser with an acceleration voltage of 20 kV.

(15) Examination, under a stereomicroscope (×50), of the crystallized matrices around a bead showed differences in crystal size and morphology between each matrix. These variations were also reflected in the dramatic differences in the intensity and shape of the signal corresponding to the peptide of interest. The matrix DHB (dihydroxybenzoic acid) which produced the largest crystals (up to 2 mm long) around the beads gave the clearest signals for the peptide and was used for all the subsequent studies.

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(20) (1) Fmoc-Ala-Leu-Phe-Leu-Ile-Thr(OBu^t)-Asp(Rink linker-Resin)-OAllyl (linked by the aspartic acid side chain to the amino functionality of the Rink amine resin. Upon cleavage from the resin the aspartyl residue is converted to an asparaginyl residue.). (2) Fmoc-Ala-Leu-Phe-Leu-Ile-Thr-Asn-Allyl ester (1053.2). (3) Fmoc-Ala-Leu-Phe-Leu-Ile-Thr-Asn-OH (1013.2). (4) H-Ala-Leu-Phe-Leu-Ile-Thr-Asn-OH (790.9). (5) Fmoc-Ala-Leu-Phe-Leu-Ile-Thr-Asn-Ser-Allyl ester (1140.3). (6) H-Ala-Leu-Phe-Leu-Ile-Thr-Asn-N(C_3H_7)-CO-NH-C_3H_7 (917.1). (7) H-Ala-Leu-Phe-Leu-Ile-Thr-Asn-NC_5H_{10} (858.0). (8) Cyclo-(Ala-Leu-Phe-Leu-Ile-Thr-Asn) (772.9). Calculated average masses are given in parentheses. Abbreviations: DIC, *N,N*-diisopropylcarbodiimide; DIEA, diisopropylethylamine; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, *N*-hydroxybenzotriazole; PyBOP, (benzotriazol-1-yloxy)trispyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid.