# Recognition Sites Incorporating Both Pyridinyl and Carboxy Functionalities Prepared by Molecular Imprinting

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Noncovalent molecular imprinting using simultaneously *two* chemically distinct functional monomers, namely the weakly basic 2-vinylpyridine and the acidic and hydrogen bonding methacrylic acid, was demonstrated in ethylene glycol dimethacrylate based copolymers. Terpolymerizations of this type were shown to be particularly useful for the preparation of imprints against carboxylic acids, which additionally contained a complex array of other chemical functionalities. Such imprinted polymers demonstrated improved recognition capabilities as compared with polymers which were prepared using only one of the functional monomers.

## Introduction

Molecular imprinting is an emerging technique for the preparation of polymers possessing highly selective recognition properties (Figure 1).[1-4] In addition to fundamental studies on molecular recognition phenomena,[5] molecularly imprinted polymers (MIPs) are recognized as being very interesting specialized separation media, especially for high-performance liquid chromatography (HPLC), in applications such as enantiomeric separations. Important examples are resolution of amino acid derivatives[6] and direct enantioseparation of drugs such as the β-adrenergic blockers.[7] In addition, investigations of regio- and enantioseparations of sugars and sugar derivatives have been studied extensively.[8] Recently it was demonstrated that under optimized conditions MIPs show binding affinities and selectivities approaching those demonstrated by antigen-antibody systems.[9] Such MIPs (as antibody mimics) were successfully applied to a new radioligand binding assay, MIA (Molecularly Imprinted sorbent Assay) for accurate determinations of drug levels in human serum.

There is a general interest in increasing the scope of imprinting both in terms of the variety of substance-types compatible with the technique and the efficiency of imprint formation. These efforts include identification of novel monomers and the development of methodologies for their use in noncovalent molecular imprinting, a technique developed and pursued in our laboratory. In addition to the widely used methacrylic acid[6,7,9] some weakly basic monomers such as *N*-vinylimidazole[10] and 2- and 4-vinylpyridine[10,11] have been used as functional monomers for molecular imprinting. Here, we demonstrate the great potential of using vinylpyridine in combination with methacrylic acid for the preparation of imprints against multifunctional print molecules. MIPs were prepared against chiral compounds and evaluated for their ability to resolve the enantiomers of the print molecule in the chromatographic mode.

## Experimental Section

All compounds were obtained from commercial sources and used as received except *N*-acetyl-D-tryptophan ethyl ester, which was synthesized from the acetyl derivative by reaction with $Cs_2CO_3$ and ethyl iodide.[12] All solvents were of either analytical or HPLC grade.

**Polymer Preparation.** A modified version of a previously developed method was employed.[6b] Print molecule, crosslinking monomer (EGDMA, 65.5 mmol, 13.0 g), and the appropriate amount of functional monomer (2VPy and/or MAA, see Tables I and II) were weighed in 50-mL borosilicate glass test tubes (Kimble, Pittsburgh, PA) and dissolved in 20 mL of acetonitrile or acetonitrile–acetone (9/1 v/v) depending on solubility of the print molecule. An amount of 150 mg of initiator (ABDV) was added, and the tubes were purged thoroughly with nitrogen before polymerization at 45 °C overnight (15 h). The resulting polymers were crushed and ground in a mechanical mortar (Retsch, Haan, FRG). Small particles (<25 μm) were collected by wet-sieving (Retsch, Haan, FRG), and grinding and sieving were repeated on the coarse particles until all polymer passed through the sieve. The fines were removed by repeated flotation in acetone.

**High Performance Liquid Chromatography.** An amount of 1.4 g of polymer particles was packed into 25 × 4.6 i.d. columns as described[6b] and washed on-line with methanol–1% triethylamine (v/v) followed by acetonitrile–1% acetic acid (v/v) until

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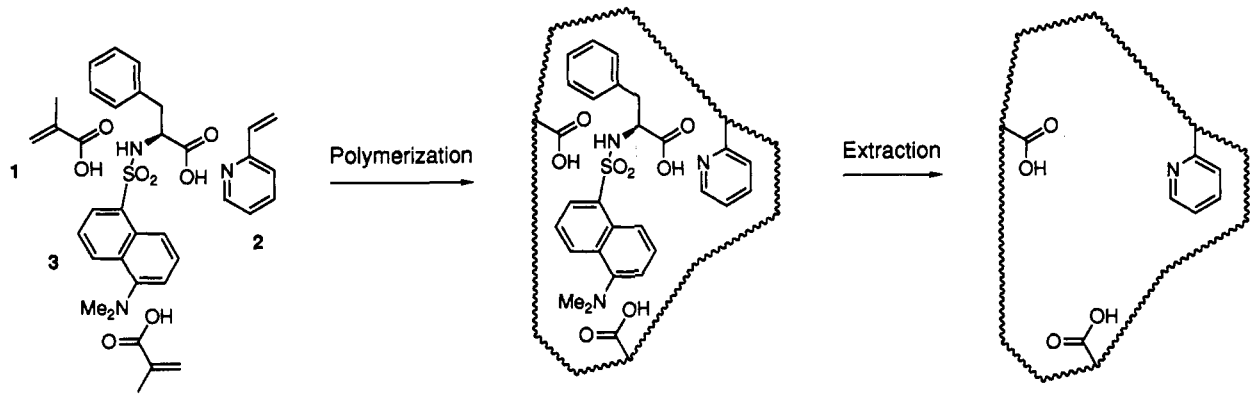


**Figure 1.** Schematic representation of imprint formation. The functional monomers, MAA (1) and 2VPy (2), are arranged around the print molecule, dansyl-L-phenylalanine (3), as a result of the noncovalent interactions between complementary chemical functionalities. After polymerization, the print molecule is removed by extraction, exposing recognition sites possessing a "memory" for the shape and chemical functionality of the print molecule.

a stable base line was obtained. Samples, consisting of 10 μg of the racemate of the print molecule in 20 μL of eluent were analyzed isocratically at a flow rate of 1 mL/min. The eluents used were (A) acetonitrile–1% acetic acid (v/v); (B) acetonitrile–0.1% acetic acid (v/v); (C) acetonitrile–chloroform-acetic acid (180:19:1, v/v/v); (D) acetonitrile–0.01% acetic acid (v/v); (E) acetonitrile–0.025% acetic acid (v/v); (F) acetonitrile–acetic acid (19:1, v/v); (G) acetonitrile; (H) chloroform–acetonitrile–acetic acid (94:5:1, v/v/v); (I) chloroform–0.25% acetic acid (v/v); (J) chloroform–0.5% acetic acid (v/v); (K) chloroform–heptane–acetic acid (500:500:1); (L) chloroform. Acetone was employed as a void marker. Capacity factor ($k'$) and separation factor ($\alpha$) were calculated using standard theory.[13] Chromatographic resolution was calculated using both a modified $R_S$ definition[8a] and an alternate model ($f/g$-values):[14] A line is drawn perpendicular to the base line from the base line through the valley (the minimum) between the peaks to a line that connects the maxima of the peaks. This distance is defined as $g$. The distance from the intersection of the two lines to the valley is defined as $f$. The resolution value ranges from 0–1, where 1.0 represents complete base-line resolution. The plate number, $N$, was calculated using $N = 5.54*(t_R/w_{1/2})\wedge 2$.[13] Frontal zone analysis was performed as described.[15]

## Results and Discussion

**Polymer Preparation.** Polymers were prepared with molecular imprints against a variety of print molecules, by copolymerization of ethylene glycol dimethacrylate (EGDMA) with methacrylic acid (MAA) and/or 2-vinylpyridine (2VPy). Three different categories of MIPs were prepared (Tables I and II): (*i*) 2VPy-MIPs using the basic monomer 2VPy as the functional monomer, (*ii*) MAA-MIPs using the acidic and hydrogen bonding monomer MAA, and (*iii*) MAA-2VPy-MIPs using both 2VPy and MAA.

According to literature data,[16] it could be expected that polymerization of a mixture of 2VPy, MAA, and EGDMA would produce terpolymers[17] where all three monomers are incorporated in an approximately random manner. Information concerning monomer reactivity ratios data was, however, not available for the extremely high cross-linking conditions normally used for molecular imprinting. Likewise, data for mixtures of more than two monomers were not available. In practice, therefore, it was only by experimental observation of the recognition properties of the resulting polymers that a judgement about the usefulness of the new monomer could be made. In initial experiments 2VPy, as well as the 4-isomer, worked well with EGDMA and MAA, as judged from the improvements in the enantiomeric resolution of Boc-tryptophan compared with the original MAA-MIPs when analyzed in the HPLC mode (data not shown). Routinely MAA-MIPs are prepared by photoinitiation at 4 °C with azobis(isobutyronitrile) (AIBN) as the initiator, since polymerization at lower temperatures usually results in improved recognition capabilities.[6b] Polymerization of batches containing 2VPy, however, was not possible under these conditions; hence, all co- or terpolymers in this study containing 2VPy were prepared by heat-induced polymerization at 45 °C using 2,2′-azobis(2,4-dimethylvaleronitrile) (ABDV) as the initiator. 2VPy-MIPs were more transparent and slightly softer than the MAA-MIPs, but were still sufficiently rigid to be analyzed under HPLC

**Table I. Chromatographic Data Obtained for MIPs Made against Boc-L-tryptophan**

| molar composition of polymer[a] | functional monomer(s) | $\alpha$ | $f/g$ | $R_S$ | eluent |
|---|---|---|---|---|---|
| terpolymers | | | | | |
| 1:2:2:20 | 2VPy/MAA | 3.67 | 0.97 | 1.9 | A |
| 1:4:4:40 | 2VPy/MAA | 3.32 | 0.94 | 2.0 | A |
| 1:8:8:40 | 2VPy/MAA | 4.35 | 1.0 | 1.9 | B |
| copolymers | | | | | |
| 1:4:0:20 | 2VPy | 2.35 | 0.95 | 1.5 | A |
| 1:0:4:20 | MAA[b] | 1.90 | 0.73 | 0.8 | C |
| reference polymers | | | | | |
| 1:4:0:20[c] | 2VPy | 1.25 | 0.61 | 0.9 | B |
| 1:0:4:20[d] | MAA | 1.0 | – | – | D |

[a] The molar composition of print molecule to 2VPy to MAA to EGDMA. [b] Data obtained from ref 6e. [c] Acetic acid (6.55 mmol) equivalent to the amount of MAA used in the terpolymers was added to the polymerization mixture. [d] Pyridine (6.55 mmol) equivalent to the amount of 2VPy used in the terpolymers was added to the polymerization mixture.

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Table II. Chromatographic Analysis of the Enantiomeric Recognition of Imprinted Polymers

| print molecule | type of polymer[a] | $\alpha$ | $f/g$ | $R_S$ | eluent |
|---|---|---|---|---|---|
| Cbz-L-Tyr-OH | MAA-2VPy[b] | 4.32 | 0.97 | 1.9 | A |
| | 2VPy | 3.81 | 0.95 | 1.9 | A |
| | MAA | 1.82 | 0.50 | – | B |
| dansyl-L-Phe-OH | MAA-2VPy[c] | 3.15 | 0.96 | 1.6 | E |
| | 2VPy | 2.20 | 0.95 | 1.5 | F |
| | MAA | 1.49 | 0.38 | 0.3 | G |
| (*R*)-phenylsuccinic acid | MAA-2VPy[b] | 3.17 | 0.91 | 1.4 | F |
| | 2VPy | 3.61 | 1.0 | 2.0 | F |
| | MAA | 1.20 | 0.11 | – | H |
| Boc-L-Phe-OH | MAA-2VPy[c] | 1.96 | 0.83 | 1.5 | B |
| | 2VPy | 1.30 | 0.56 | 0.9 | B |
| | MAA[d] | 1.77 | 0.92 | 1.4 | I |
| (*R*)-(−)-mandelic acid | MAA-2VPy[c] | 1.50 | 0.4 | – | A |
| | 2VPy | 1.20 | 0.51 | 0.7 | A |
| | MAA[d] | 1.43 | 0.81 | 1.1 | J |
| Ac-L-Trp-OEt | MAA-2VPy[c] | 1 | – | – | K |
| | 2VPy | 1 | – | – | G |
| | MAA[e] | 3.92 | 1.0 | 2.2 | L |

[a] Molar compositions as in Table I unless otherwise stated. [b] A molar composition of 1:8:8:40 was used, see Table I. [c] A molar composition of 1:4:4:40 was used, see Table I. [d] Data obtained from ref 6e. [e] This polymer was prepared against Ac-D-Trp-OEt using chloroform as the solvent of polymerization.

conditions. The appearance of MAA–2VPy-MIPs was similar to the MAA-MIPs.

**Chromatographic Experiments.** The enantioselective recognition properties of the MIP preparations were analyzed by HPLC, as has been described previously for other imprinted polymers.[6] The separation factors ($\alpha$) and resolution values obtained are presented in Tables I and II. The eluent composition was optimized individually for each polymer preparation to give a capacity factor ($k'$) of the least retained enantiomer in the range of 1–5. The plate numbers, $N$, calculated for a nonretarded, noninteracting void marker (acetone), were generally in the range of 6000 to 12000 $m^{-1}$. $N$ is a measure of column performance, and the values obtained are good for particles of the size and quality used (25 $\mu$m and nonspherical particles).[13]

The use of 2VPy as functional monomer in molecular imprinting was investigated, both as single functional monomer and in combination with MAA, using Boc-L-tryptophan as the print molecule (Table I). MAA-MIPs made against Boc-L-tryptophan have been demonstrated previously.[6e] The 2VPy-MIP resolved the enantiomers of Boc-Trp-OH more efficiently than the MAA-MIP {*i.e.* higher separation factors ($\alpha$) were obtained}. The MAA–2VPy-MIP showed even higher enantiomeric separation power. Three types of terpolymers were prepared where variations in the molar ratio of functional monomer to print molecule were made (Table I). The polymer prepared with the highest molar ratio of functional monomer to print molecule gave the best separation ($\alpha$ = 4.35). This polymer completely resolved the applied amount of racemate (33 nmol, 10 $\mu$g) ($f/g$ = 1.0). The number of accessible binding sites, determined by frontal zone analysis were for 2VPy-MIP, 35 ± 4.1 $\mu$mol/g; MAA-MIP, 10.6 ± 2.3 $\mu$mol/g; and 2VPy–MAA-MIP, 17.6 ± 3.2 $\mu$mol/g of dry polymer.

In order to verify the cooperative action of the functional monomers in the terpolymer, reference polymers were made where pyridine and acetic acids were substituted for 2VPy and MAA, respectively. In both cases, these reference polymers showed much lower enantioselectivity than was recorded for the terpolymer and each copolymer (Table I). Addition of a second functional monomer to the polymerization mixture of either of the copolymers leads to a polymer showing increased enantiorecognition. Addition of pyridine or acetic acid, nonmonomeric analogues of the second monomer, instead leads to a polymer which exhibits decreased recognition capabilities as compared with the parent copolymer. These results are indicative of a concerted interaction of both functional monomers with the print molecule in the prepolymerization mixture.

2VPy-MIPs were shown to be excellent for enantiomeric recognition of compounds containing carboxylic acid functionalities (Table II). For example, separation of the enantiomers of Cbz-tyrosine and phenylsuccinic acid were efficient on their corresponding MIPs ($\alpha$ = 3.81 and 3.61, respectively). The enantiomeric resolution values were high, *e.g* the enantiomers of phenylsuccinic acid were completely resolved ($f/g$ = 1.0). 2VPy-MIPs could be prepared against other carboxylic acid derivatives with similar results (Table II). Enantioselective recognition of the ethyl ester of acetyltryptophan, however, was not possible on this type of MIP. Since this print molecule lacked a free carboxylic acid functionality, interaction with 2VPy in the polymerization mixture was probably to weak to allow the formation of imprints.

MAA-MIPs have previously been studied in several thorough investigations and their operation is to some extent understood.[6,18] This present study confirms and extends the previous results. The separation factors obtained for MAA-MIPs were generally in the range of 1.2–1.9 and the resolutions were moderate (Table II).

In most instances, MAA–2VPy-MIPs performed remarkably well in the resolution of the enantiomers of the print molecule. The racemates of Boc-tryptophan, Cbz-tyrosine, dansylphenylalanine, and phenylsuccinic acid were all resolved with very high separation factors ($\alpha$ > 3) and resolution values ($f/g$ = 0.9–1.0). Contrary to these findings, but analogous to the corresponding 2VPy-MIP, the MAA–2VPy-MIP was not able to separate the enantiomers of acetyltryptophan ethyl ester.

In this report we demonstrate the possibility of simultaneously using two chemically distinct functional monomers, namely 2VPy and MAA, for noncovalent molecular imprinting. Such imprinted polymers demonstrate improved recognition capabilities as compared with the corresponding 2VPy- or MAA-MIPs. We believe that this approach can be extended to the use of a multitude of monomers each with different functionality, resulting in greatly enhanced versatility of noncovalent imprinting.

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