Towards Covalent Vaccination

**IMPROVED POLYCLONAL HIV NEUTRALIZING ANTIBODY RESPONSE INDUCED BY AN ELECTРОPHILIC gp120 V3 PEPTIDE ANALOG**

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Rare monoclonal antibodies (Abs) can form irreversible complexes with antigens by enzyme-like covalent nucleophile-electrophile pairing. To determine the feasibility of applying irreversible antigen inactivation by Abs as the basis of vaccination against microbes, we studied the polyclonal nucleophilic Ab response induced by the electrophilic analog of a synthetic peptide corresponding to the principal neutralizing determinant (PND) of human immunodeficiency virus type-1 (HIV) gp120 located in the V3 domain. Abs from mice immunized with the PND analog containing electrophilic phosphonates (E-PND) neutralized a homologous HIV strain (MN) ~50-fold more potently than control Abs from mice immunized with PND. The IgG fractions displayed binding to intact HIV particles. HIV complexes formed by anti-E-PND IgG dissociated noticeably more slowly than the complexes formed by anti-PND IgG. The slower dissociation kinetics are predicted to maintain long-lasting blockade of host cell receptor recognition by gp120. Pre-treatment of the anti-PND IgG with a haptenic electrophilic phosphonate compound resulted in more rapid dissociation of the HIV-IgG complexes, consistent with the hypothesis that enhanced Ab nucleophilic reactivity induced by electrophilic immunization imparts irreversible character to the complexes. These results suggest that electrophilic immunization induces a sufficiently robust nucleophilic Ab response to enhance the anti-microbial efficacy of candidate polypeptide vaccines.

Antibodies (Abs) that bind human immunodeficiency virus type 1 (HIV) with irreversible character are conceptually analogous to reagents with infinite binding affinity. Rare monoclonal Abs can form unusually stable immune complexes expressing covalent character (1, 2). The combining site of such monoclonal Abs is usually intended to replicate enzyme active sites. Immunization with an Ab to β-lactamase, for example, is reported to induce an anti-idiotypic monoclonal Ab that forms a covalent intermediate with a β-lactam compound that is sufficiently stable to be detected in denaturing electrophoresis gels (1). Monoclonal Abs raised to an analog of HIV gp120 containing electrophilic phosphate diesters (E-gp120) form noncovalent immune complexes that are subsequently converted to irreversible complexes by nucleophile-electrophile interactions (2). In this example, stimulation with the electrophilic groups is suggested to strengthen the nucleophilic reactivity of Ab combining sites by the adaptive immunological processes that are also responsible for improved noncovalent binding, i.e. V-(D)-J gene recombination, somatic hypermutation and combinatorial diversification. B cell clonal selection is thought to be driven by binding of antigen to the B cell receptor (BCR), i.e. surface Ig complexed to signal transducing proteins. As irreversible binding should permit prolonged BCR occupancy, improvement of the nucleophilic reactivity over the course of adaptive B cell differentiation is feasible. The nucleophilic reactivity is reminiscent of enzymatic active sites, in which activated groups formed by intramolecular hydrogen bonding interactions acquire an ability to conduct nucleophilic attack at electron-deficient sites in substrates. For example, the nucleophilic reactivity of the Ser-His-Asp triad in serine proteases results in the formation of enzyme-substrate covalent reaction intermediates (3). Indeed, similar nucleophilic triads have been identified in monoclonal Ab combining sites by crystallography and mutagenesis studies (4–6). Completion of the catalytic cycle following the nucleophile-electrophile reaction requires various additional accessory groups in the active site. Consequently, some but not all nucleophilic Abs proceed to catalyze chemical reactions (7).

The ability to form long-lasting immune complexes can be anticipated to enhance the antigen inactivation potency of irreversibly binding Abs compared with their reversibly binding counterparts. This leads to the hypothesis that electrophilic antigen analogs can serve as the basis for vaccine formulations capable of inducing improved protective Ab responses to microbial antigens compared with conventional vaccines. The hypothetical improvement in vaccine formulation will depend...
on the proportion of the induced polyclonal Ab response that displays covalent character while maintaining the correct epitope specificity necessary for recognition of the native antigen structure. In previous studies using full-length E-gp120 as the immunogen, several monoclonal Abs were identified that formed unusually stable immune complexes with gp120 devoid of exogenously introduced electrophilic groups (2). However, full-length E-gp120 expresses a multitude of epitopes, and we were unable to relate Ab covalency and HIV neutralization because of the varying Ab epitope specificities. Moreover, a rigorous covalent ELISA protocol was employed to screen hybridoma supernatants. Consequently, there is no assurance that anything more than a small minority of the overall Ab response to E-gp120 expresses the desired covalent character of the monoclonal Abs.

The immune response following HIV infection is dominated by Abs to the principal neutralization determinant (PND) of gp120 corresponding to residues 306–328 located in the V3 domain (8–10). Immunization with the synthetic PND peptide induces Ab responses that neutralize HIV strains with sequences similar to the PND immunogen (11–14). Here, we studied the comparative HIV neutralizing and binding characteristics of polyclonal Ab preparations induced by an electrophilic analog of the PND peptide (E-PND) and the control PND peptide devoid of exogenously introduced electrophiles. We observed that immunization with E-PND induced Abs that neutralized HIV more potently and dissociated from intact HIV virions more slowly than control Abs from PND-immunized mice. These results indicate the improved antigen inactivation potency due to Ab nucleophilicity and suggest the utility of electrophilic immunization as a novel vaccination strategy.

**EXPERIMENTAL PROCEDURES**

**PND Analogs**—PND peptide 1a corresponding to gp120 residues 306–328 of HIV strain MN (YNKRRKHIHGPRAGYTTPKNIIG) and its biotinylated analog (Bt-PND 1b) were prepared by Fmoc-based solid phase synthesis followed by purification with reversed phase HPLC (1a: observed m/z 2705.3; calcd m/z 2705.2. 1b: observed m/z 3043.0; calcd m/z 3044.6; Genemed Synthesis, South San Francisco, CA). The electrophilic phosphonate analog of PND (E-PND 2a) was prepared by acylation of 1a with N-hydroxysuccinimidyl ester of diphenyl (suberoyl)amin(4-aminophenyl)methane phosphonate as follows. PND 1a (10 mg, 37 µmol) was allowed to react with the acylating agent (33 mg, 44 µmol) in dimethyl sulfoxide (6.6 ml) and 100 mM phosphate-buffered saline, pH 8 (4.0 ml) for 1 h. Excess acylating agent was quenched by addition of 1 M glycine (2 ml). 15 min thereafter, the precipitate was collected by centrifugation, washed with cold water (4 ml x 3), and subjected to HPLC purification (YMC-Pack ODS-AM, 4.6 x 250 mm; gradient of 10% to 80% acetonitrile in 0.05% trifluoroacetic acid/water, 45 min). This yielded chromatographically pure E-PND 2a (5.4 mg, 31%), which was characterized by electrospray ionization mass spectrometry (observed m/z, 1594.5, 1196.5, 957.7; calcd m/z for C_{235}H_{319}N_{51}O_{48}P_{4}S, 1595.1 (3+), 1196.6 (4+), 957.5 (5+)). Biotinylated E-PND (Bt-E-PND 2b) was prepared from Bt-PND 1b in the same manner (observed m/z, 1151.1, 921.3, 767.9; calcd m/z for C_{223}H_{314}N_{51}O_{48}P_{4}S, 1151.6 (4+), 921.5 (5+), 768.0 (6+).

**Antibodies**—Female MRL/lpr mice (n = 5/immunogen; Jackson Laboratory, Bar Harbor, ME; 8 weeks age) were immunized intraperitoneally on days 0, 15, 29, 44, 58, and 101 with PND 1a or E-PND 2a (50 µg for the first 4 injections and 200 µg for the last 2 injections) in Ribi adjuvant (monophosphoryl lipid A-trehalose dicorynomycolate emulsion; Sigma-Aldrich). Blood was obtained from the retroorbital plexus over the course of the immunization schedule (days 0, 15, 29, 44, 58, 65, and 111). Development of PND-reactive IgG was examined by ELISA using Bt-PND 1b (4 µg/ml) immobilized on streptavidin-coated plates, sera diluted 1:5000 in 10 mM PBS containing 0.025% Tween 20 and 1% bovine serum albumin, and peroxidase-conjugated goat anti-mouse IgG (Fc specific; Sigma-Aldrich) as secondary Ab. IgG was purified to electrophoretic homogeneity from serum (prepared from blood collected 10 days after the last immunization) by affinity chromatography on protein-G Sepharose (GE Healthcare, Piscataway, NJ) (15).

**HIV Neutralization**—Neutralization of HIV (strain MN, clade B) by serially diluted sera or purified IgG samples was determined in a “microplate” reduction assay using cells of the MT-2 T lymphocyte cell line as hosts (16). Neutralization of strain ZA009 (clade C) was measured by the p24 capsid protein assay with human peripheral blood mononuclear cells as hosts (17). Concentrations yielding 50 and 80% inhibition (IC_{50} and IC_{80}) were obtained from the least-square-fits to a sigmoidal dose-response shown in Equation 1.

\[
%\text{Neutralization} = \frac{\text{TOP} - \log IC_{50} - X \times \text{HillSlope}}{\text{TOP} - \log IC_{80}}
\]

(Eq. 1)

**HIV Binding Assays**—Purified IgG (17 µg/ml) and HIV (MN strain, 1.6 x 10^{6} TCID_{50}/ml; TCID_{50} 50% tissue culture infective dose) were incubated in a mixture of 10 mM phosphate-buffered saline, pH 7.4, and RPMI1640 (1:1) containing 10% fetal bovine serum at 4 °C for 16 h. HIV-IgG complexes (and free IgG) were captured on Protein G-Sepharose (100 µl of settled gel) using Bio-Spin chromatography columns (Bio-Rad), and unbound HIV removed by washing with the reaction buffer (500 µl x 8). The captured complexes were eluted with 100 mM glycine-HCl, pH 2.7 (400 µl), HIV was lysed with Triton X-100 (1%), and p24 in the lysates was measured with Coulter HIV-1 p24 Antigen Assay kit. Values were corrected for nonspecific HIV binding to the affinity gel, determined by identical processing of control HIV treated with diluent in the absence of IgG (A490, 0.21 ± 0.01). In competition experiments, IgG and HIV were incubated in the presence of excess PND peptide 1a (50 µg/ml) or an irrelevant peptide (gp120 residues 465–479 of MN strain; NIH AIDS Research and Reference Reagent program), and HIV-IgG complexes were measured as above. To determine dissociation kinetics, HIV-IgG complexes were allowed to form for 16 h (IgG 50 µg/ml, HIV 1.6 x 10^{6} TCID_{50}/ml), excess PND 1a (50 µg/ml) was added to the reaction mixtures to preclude reassociation of any complexes undergoing dissociation. Aliquots of the reaction mixtures withdrawn 0.5, 2, 4, 8, and 14 h thereafter were immediately subjected to pro-
tein G chromatography and p24 assays to determine residual immune complexes as described above.

**IgG Nucleophilic Reactivity**—Bt-E-PND 2b (1 μM) was incubated with IgG (75 μg/ml) for 2 h, the reaction mixtures were boiled, subjected to reducing SDS-electrophoresis, and the covalent adducts were detected by streptavidin-peroxidase staining of the blots as described previously (18). To study the role of IgG nucleophilic reactivity in HIV binding, the IgG (1.4 mg/ml) was treated with the haptenic phosphate 3 (1 mM; diphenyl benzylxocarbonylamino(4-amidinophenyl)methanephosphonate, synthesis described in ref 19) at room temperature for 13 h. After removing unreacted phosphate by gel filtration (Bio-spin 6, Bio-Rad; IgG recovery 88%), the dissociation kinetics of the HIV-IgG complexes was studied as above.

**RESULTS**

**Immunogenicity**—The E-PND immunogen 2a corresponds to the amino acid sequence of HIV gp120 residues 306–328 (MN strain) with 4 electrophilic phosphate diester groups located at the side chains of Lys308, Lys310, and Lys324 residues and the N terminus (Fig. 1A). The phosphate groups are structurally identical to those incorporated into full-length gp120 and other polypeptides in previous studies (7, 18, 20–22). The resultant electrophilic polypeptide bind nucleophilic sites in Abs covalently (18, 20–22). We employed multiple phosphate groups within a single E-PND molecule to increase the probability of nucleophile-electrophile pairing coordinated with noncovalent binding at the proximate peptide regions. The control immunogen PND 1a was the identical peptide structure devoid of the phosphate groups. Successive immunizations of mice with E-PND 2a or PND 1a resulted in progressively increasing PND binding activity in serum IgG obtained over days 15 to 65 (mean A490 ± S.D. for 1:5000-diluted pooled sera using immobilized Bt-PND 1b increased from 0.06 ± 0.10 to 0.71 ± 0.13 in 2a-immunized mice and from 0.01 ± 0.01 to 0.42 ± 0.28 in 1a-immunized mice; n = 5 mice each; preimmune mouse serum binding, 0.01 ± 0.01). ELISA assays using the final bleeds obtained on day 111 indicated that each of the mice had mounted an IgG response to PND (A490 values for sera from individual 2a-immunized mice at 1:1000 dilution, 0.79 ± 0.01, 0.47 ± 0.03, 1.48 ± 0.01, 0.72 ± 0.02, 2.59 ± 0.09). These results indicate that Abs to E-PND can recognize PND devoid of the electrophilic groups.

**HIV Neutralization**—Pooled sera from the mice (day 111) were assayed for the ability to neutralize HIV strain MN (clade B, coreceptor CXCR4-dependent) using MT-2 host cells. Serially diluted sera were incubated with the virus and the infectivity was measured by the microplaque assay (16). Dose-dependent HIV neutralization was observed, with the sera from E-PND 2a-immunized mice displaying 44–272-fold greater neutralizing potency compared with sera from PND 1a-immunized mice (dilution yielding 50% neutralization, 1:787 versus 1:34,444; dilution yielding 80% neutralization, 1:54 versus 1:14,692; Fig. 1B). The consistency of the Ab response was confirmed by assaying by sera from individual E-PND 2a-immunized mice and PND 1a-immunized mice (Fig. 1C; n = 5 and 4 mice, respectively). Mean % neutraliza-
greater than control IgG from preimmune mice (Fig. 2A). In the presence of excess PND peptide, complete competitive inhibition of the binding was observed in all assays. After the complexes had been formed, excess PND peptide was added to the assay mixtures to assess the dissociation rates of the HIV-IgG complexes. Following treatment with phosphonate, and removal of the unreacted phosphonate, a subpopulation that did not dissociate detectably between 8 and 18 h (∼30% of initial complexes).

Ab Nucleophilic Reactivity—The strongly electrophilic phosphonate in probe E-PND 2b is known to form stable covalent bonds with Ab nucleophiles (7, 18, 20–22). In addition, this probe contains the PND peptide regions available for noncovalent Ab binding. As a test of antigen-specific nucleophilic reactivity, we measured the formation of covalent adducts of IgG and Bt-E-PND 2b. Boiled reaction mixtures of the IgG and E-PND 2b were analyzed by reducing SDS-electrophoresis and densitometry. Adducts of 2b formed by the IgG and light chains from E-PND-immunized mice accumulated more rapidly than the adducts from control PND-immunized mice or control non-immune IgG (Fig. 4A, Fig. 4B, and Fig. 4C). These results indicate that the antigen-specific binding affinity of covalent adducts is higher in E-PND-immunized mice than in control PND-immunized mice or control non-immune IgG.

To study immune complex stability, we measured the dissociation rates of the HIV-IgG complexes. After the complexes had been formed, excess PND 1a (18.5 μM) was added to the reaction mixtures to preclude reassociation of HIV that had undergone dissociation, and the residual complexes were measured periodically by the p24 assay. Dissociation of HIV complexes to IgG induced by immunization with PND 1a occurred rapidly and proceeded at a rate consistent with the first-order dissociation equation (half-life, t½, 10.6 min; r² 0.985; Fig. 3). Dissociation of anti-E-PND IgG complexes was substantially slower and suggested two subpopulations of complexes, a subpopulation that dissociated slowly over 8 h (∼70% of initial complexes; nominal t½, assuming first order kinetics and complete dissociation over 8 h, 2.5 h, r² 0.971) and another subpopulation that did not dissociate detectably between 8 and 14 h (∼30% of initial complexes).
the control HIV-IgG complexes, the dissociation of HIV complexes formed by phosphonate 3-treated IgG proceeded to near-completion within 1 h (Fig. 4B). These results suggest that nucleophilic sites in IgG from the E-PND-immunized mice are responsible for the enhanced stability of the immune complexes and superior HIV neutralization.

DISCUSSION

An effective vaccine against diverse HIV strains found in different geographical regions and escape mutants that emerge in the course of infection remains elusive. The anti-viral efficacy of various Abs directed to the neutralizing epitopes of the HIV envelope depends largely on their first order dissociation rate constants (k_{off}/t^{1/2} = 1/k_{on}) and concentrations in biological fluids. The Ab-virus association rate constant (k_{on}) also contributes to the observed binding affinity (K = k_{on}/k_{off}), but different Abs to a given viral epitope usually display comparable k_{on} values, as this constant is controlled mainly by the rate of diffusion and the orientation of collisions between the reactants. A previous study has highlighted the correlation between the HIV neutralizing potency and dissociation rate constants of monoclonal Abs to the PND, the epitope targeted in the present study (26). We reported that rare monoclonal Abs raised to an electrophilic analog of full-length gp120 neutralized HIV and formed unusually stable immune complexes with gp120 devoid of exogenous electrophiles (2). The stability of the immune complexes was attributable to the adaptively strengthened nucleophilic groups in the Abs induced by electrophilic immunization. The nucleophilic groups are hypothesized to lend covalent character to the complexes by pairing with natural electrophilic groups in gp120. Regrettably, polyclonal IgG from the mice immunized with full-length E-gp120 failed to neutralize HIV at levels superior to IgG from non-immunized mice, presumably because the overall polyclonal immune response is dominated by Abs to irrelevant epitopes and Abs with sufficient nucleophilic reactivity to the neutralizing epitope were present only at low concentrations.

In the present study, a well characterized synthetic analog of the peptide corresponding to the principal neutralizing determinant of HIV strain MN was studied as immunogen (E-PND). Four electrophilic phosphonates were incorporated within the 23 residues peptide analog to maximize the opportunity of B cell adaptive differentiation in response to electrophilic stimulation. Polyclonal Ab preparations obtained following E-PND immunization formed complexes with intact HIV virions. The complexes were poorly dissociable or not at all dissociable. The polyclonal anti-E-PND IgG neutralized HIV ~50-fold more potently than control Abs to PND devoid of phosphonate electrophilic groups. This is consistent with the prediction that slower dissociation of the HIV-IgG complexes should prolong the duration over which the Ab-complexed virus exists in non-infectious form. Biochemical analysis confirmed the enhanced nucleophilic reactivity of the anti-E-PND Abs and the importance of the nucleophilic reactivity in prolonging immune complex longevity. It may be concluded that the nucleophilic Abs responsible for forming stable immune complexes are present in the polyclonal IgG mixtures at concentrations sufficient to achieve functionally useful viral inactivation.

Concerning epitope specificity, the PND (residues 306–328) is located in the highly mutable V3 region of gp120 (12). Consistent with the neutralization results for strains MN and ZA009 in the present study, Abs to the V3 region display typespecific neutralizing activity, that is, they neutralize the infecting HIV strain, but V3 sequence mutants resistant to infection emerge following infection (11–14). The mechanism of neutralization by anti-PND Abs is thought to involve sterically hindered recognition of gp120 by HIV coreceptors CCR5 and CXCR4 expressed by host cells (27–29). In particular, residues Pro^316^-Arg^318 are reported to be important for CCR5 binding (30–32). Attempts to induce broadly neutralizing Abs that recognize diverse HIV strains using mixtures of synthetic peptides with varying V3 sequences have been reported (e.g. Refs. 33, 34). However, V3 sequence diversity is so great that a large library of immunogenic peptides is necessary to justify hopes of inducing a broadly neutralizing Ab response. For example, the 201 known clade B virus strains available in the Los Alamos database contain 179 distinct PND sequences. Monoclonal Abs that recognize the more conserved Gly-Pro-Gly-Arg sequence within the PND are reported to neutralize various HIV strains comparatively broadly (35, 36). To the extent that inducing a
broadly neutralizing Ab response to the PND or regions within the PND is feasible by these means, our results suggest that inclusion of electrophilic groups in the immunogenic peptide (or combination of peptides) will be helpful to increase the potency of neutralization. In principle, the electrophilic immunization strategy is applicable to targeting of any peptide epitope. gp120 also contains important neutralizing epitopes outside the PND, e.g., the conserved regions that participate in binding to host cell CD4 receptors (37). However, these epitopes are generally poorly immunogenic and they are composed of peptide regions distant in the linear sequence of the protein (conformational epitopes). No linear gp120 peptide or mimic is available presently that reproducibly induces the synthesis of broadly neutralizing Abs to the diverse HIV strains responsible for the pandemic.

The following empirical and theoretical points are relevant in assessing the potential generality of the electrophilic immunization approach: (a) Electrophilic phosphonates were originally developed as covalent inhibitors of the catalytic sites of serine proteases (38). Haptenic phosphonates react covalently with all Ab preparations examined thus far, including monoclonal Abs, single chain Fv constructs (Vλ and VH domains tethered by a linker) (18, 19, 23) and Igs contained in BCRs (39). This suggests that the nucelophilic reactivity is ubiquitously distributed in Ab combining sites regardless of noncovalent binding specificity. (b) Noncovalent epitope recognition is reported to accelerate the covalent reaction of electrophilic phosphate groups incorporated into several polypeptide with various polyclonal and monoclonal Abs specific for these polypeptides (18, 20–22). It appears, therefore, that the noncovalent and nucleophilic Ab subsites are within sufficient proximity to express their functions in a coordinated manner. (c) Only a small subset of nucelophilic Abs displays the ability to hydrolyze peptide bonds (7). This may be understood from the requirement for additional rate-limiting events for completing the catalytic cycle following the initial nucleophilic attack step, i.e., water attack and product release. We did not detect PND hydrolysis IgG from E-PND immunized mice (4). Importantly, the electrophilic phosphate is predicted to favor adaptive strengthening of Ab nucleophilicity, but the immunogen lacks structural elements that can induce the synthesis of Abs capable of completing the catalytic cycle; and (d) Observations using noncatalytic monoclonal Abs raised to full-length E-gp120 suggest the feasibility of immune complex stabilization by resonant nucleophilic Ab subsites are within sufficient proximity to express their functions in a coordinated manner. (c) Only a small subset of nucelophilic Abs displays the ability to hydrolyze peptide bonds (7). This may be understood from the requirement for additional rate-limiting events for completing the catalytic cycle following the initial nucleophilic attack step, i.e., water attack and product release. We did not detect PND hydrolysis IgG from E-PND immunized mice (4). Importantly, the electrophilic phosphate is predicted to favor adaptive strengthening of Ab nucleophilicity, but the immunogen lacks structural elements that can induce the synthesis of Abs capable of completing the catalytic cycle; and (d) Observations using noncatalytic monoclonal Abs raised to full-length E-gp120 suggest the feasibility of immune complex stabilization by resonant nucleophilic-electrophile-pairing at the naturally occurring electrophilic reaction centers in the polypeptide antigen, e.g., the carbonyl groups of backbone peptide bonds or side chain amide bonds in structure IC’ , Fig. 4C (2). Such interactions are hypothesized to impart partial covalent character to the complexes and impede their dissociation. Similar structures are thought to exist in the transition state of enzymatic reactions (3). To our knowledge there is no theoretical bar to stabilization of ground state protein-protein complexes by this mechanism. B cell clonal selection processes favor increased BCR occupancy by the antigen. Covalent binding of electrophilic phosphonates due to improved BCR nucleophilic reactivity is predicted to be an immunological selectable event, and the desirable consequence of the improved nucleophilicity is the ability to bind the target polypeptide antigen with covalent character.

In summary, our studies using the model E-PND immunogen indicate that electrophilic immunization induces a robust polyclonal nucleophilic Ab response with improved viral binding and inactivation potency. Concerning HIV vaccine development, the caveat remains that PND sequence divergences may limit the functional efficacy of E-PND immunization. However, the E-PND studies validate electrophilic immunization as a potentially general approach that can be applied to induce adaptive Ab responses capable of binding microbial antigens with irreversible character and help improve vaccine efficacy.

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Footnotes

4 Incubation of PND 1a (0.1 mM) in the presence of IgG (1 μM) from hyperimmune mice for 3 h did not result in detectable product accumulation at levels greater than background in control incubations conducted using an equivalent concentration of preimmune IgG determined by reversed-phase HPLC with an electrospray mass detector (Vydac C18 MASS SPEC column, 2.1 x 150 mm; 3–60% acetonitrile in 0.1% formic acid and water; retention time of intact PND 1a, 14.3 min).
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