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Bioorganic studies of transmitter receptors with philanthotoxin analogs

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Abstract: *Philanthotoxin-433 (PhTX-433)*, isolated from the venom sac of the wasp *Philanthus triangulum*, is a noncompetitive antagonist of glutamate and nicotinic acetylcholine receptors. Extensive structure-activity studies performed with approximately 100 synthetic analogs have provided several highly active analogs carrying photolabile/radioactive groups at various sites of this linear molecule. Preliminary photoaffinity labeling studies of a nicotinic acetylcholine receptor have led to a model showing the mode of binding of PhTX to this receptor.

INTRODUCTION

Piek and co-workers reported that the venom of *Philanthus triangulum F.*, a digger wasp found in the Saharan desert that preys on honeybees, blocks the quisqualate-sensitive glutamate receptor (qGlu-R) located post-junctionally on locust muscle (ref. 1). The most active component of the venom, δ-philanthotoxin (δ-PhTX), was isolated from the venom glands of the female wasp and assigned the structure shown in Fig. 1, with the butyryl/tyrosyl/thermospermine sequence, from spectral data and synthesis of the racemic toxin (ref. 2). We independently isolated the toxin using a honeybee worker paralysis assay and a locust neuromuscular preparation, and determined that the structure of the toxin is PhTX-433 (numerals denote the number of methylenes in the polyamine) as shown with the *S* configuration, based on CD spectroscopy and synthesis (ref. 3). PhTX-433 is an efficient, non-competitive, reversible antagonist of qGlu-R of locust skeletal muscle and some vertebrate Glu-R that is thought to act by open channel block. About 40 polyamine toxins, some of which are shown in Fig. 1, have also been isolated from spider venoms and also found to be potent antagonists of qGlu-R (refs. 4-8).

Members of the Glu-R family are major excitatory receptors in the vertebrate central nervous system (CNS) as well as in the insect brain and peripheral nervous system, where they regulate synaptic transmission. They may also be involved in neuronal cell degeneration in the vertebrate CNS. Excitation of some Glu-R induces not only a short-term depolarization but also a long-term potentiation (LTP), which has been proposed as a mechanism for information storage in the brain. Glu-R belong to a super-family of ligand-gated, ion channel receptors which includes nicotinic acetylcholine receptors (nACh-R) and γ-aminobutyric acid receptors (GABA-R).

Vertebrate Glu-R are classified into two sub-families: ligand-gated ion channel (ionotropic) and G-protein coupled (metabotropic) receptors. The ionotropic receptors are activated by L-glutamate (Glu) binding and elicit the transport of Na^+ and Ca^{++} into the cell and K^+ out of the cell. The ionotropic Glu-R are further divided into two sub-classes based on their responses to exogeneous ligands: (i) N-methyl-D-aspartate receptors (NMDA-R) and (ii) kainate (KAIN) / α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (non-NMDA-R). The vertebrate

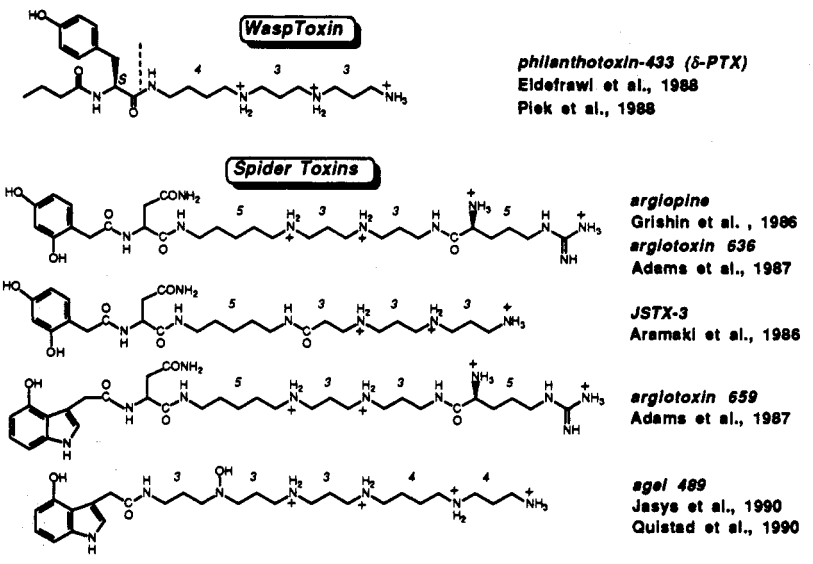

Figure 1.

metabotropic Glu-R are activated by quisqualate, which enhances inositol phosphate metabolism. Invertebrate Glu-R consists of ionotropic receptors classified into three subtypes: (i) quisqualate receptors (qGlu-R, which gate cation channels), (ii) ibotenate receptors (which gate Cl^- channels), (iii) a purported KAIN-R, (iV) and a purported NMDA-R. It was originally thought that PhTX was specific for the invertebrate qGlu-R but instead, PhTX and analogs turned out to be rather nonspecific and have affinity for nACh-R as well as for many types of Glu-R; compared to PhTX analogs, the structurally more complex spider toxins appear to be relatively more specific for vertebrate NMDA-R. Numerous analogs of spider and wasp toxins have been synthesized worldwide in order to develop a new class of pharmaceutical drugs or novel insecticides by increasing their specificity toward various receptor subtypes. The focus of our research has been to take advantage of the unique linear structures of the analogs and prepare photolabile bioactive derivatives and use them to investigate the tertiary structure of the large membrane-bound transmitter receptor proteins by photoaffinity mapping and other techniques.

STRUCTURE/ACTIVITY STUDIES (SAR)

Several groups have reported the preparation of PhTX analogs of higher activity than the native PhTX-433 (ref. 9-11). We have prepared approximately 100 analogs of PhTX-433 in a systematic manner by dividing the molecule into four regions, I ~ IV, performed single and multiple structural modifications and have assayed for binding to qGlu-R, nACh-R and NMDA-R. While, for qGlu-R, the relative potencies of the series PhTX-433 (natural product), -343 and -334 are 1.3, 1.0 and 1.5, respectively, PhTX-343 containing a symmetric polyamine moiety was selected as the basic structure used for modifications due to it's easy synthesis. With a few exceptions, analogs were prepared by a convergent pathway following straightforward routes (ref. 12); hybrids between PhTX and argiotoxins (ref. 13a) and PhTX analogs with side-chains extending from the Region I methylene groups (see "Bu-433" in Fig. 2) have also been prepared (ref. 13b). Assays of about 60 of the analogs on qGlu-R resulted in the discovery of several analogs of high potency (ref. 14), but unfortunately other studies showed that PhTX and analogs are also active against other types of ionotropic Glu-R (ref. 15, 16) as

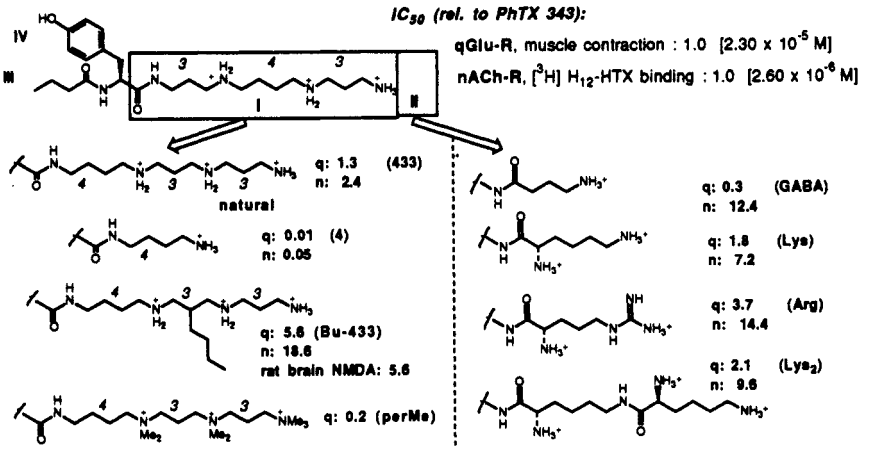

Figure 2.

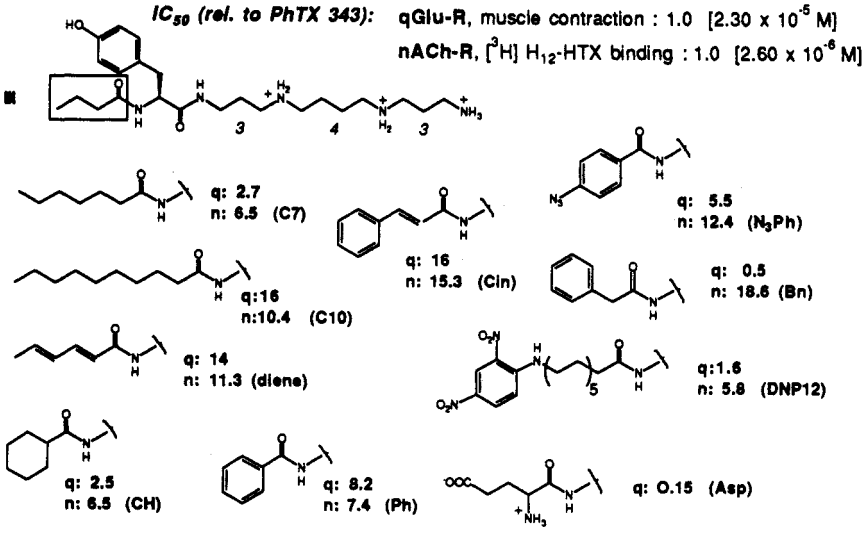

Figure 3.

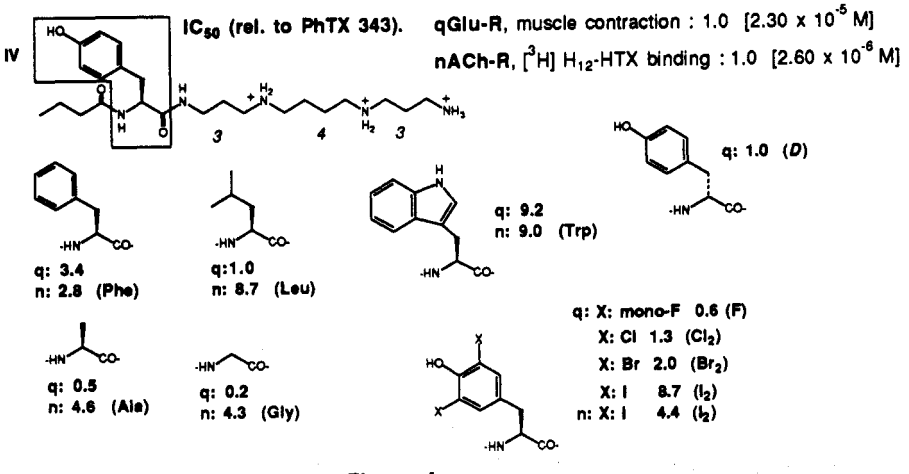

Figure 4.

well as nACh-R (refs. 16, 17). The nonspecificity of PhTX analogs makes them poor ligands for receptor protein isolation; on the other hand, the availability of photoaffinity labeled toxins, as well as the ease of preparation of radiolabeled toxins makes them suited for receptor isolation from relatively pure sources, a process which is in use in our laboratory (unpublished). More importantly, the linear, flexible and extended structure of PhTX's makes them uniquely suited for tertiary structural studies of PhTX receptor binding sites.

Since none of the Glu-R have as yet been overexpressed in quantities sufficient for biochemical structural studies, the following discussion is centered on the nACh-R of the electric ray *Torpedo californica,* which is available in mg quantities. nACh- and Glu-Rs share some homology in the amino acid sequences of the transmembrane segments constituting their purported channels. The fact that the SAR results show general similarities among the various receptors (Figs. 2-4) leads to the following important conclusions: (i) the mode of binding of PhTX analogs to nACh-R is similar in other receptors; (ii) the uniquely intermixed hydrophilic and hydrophobic structural moieties of the various PhTX analogs tested suggest that the general topology of the transmembrane M2 segments purportedly constituting the channels in other receptors shares similar structural aspects as that of nACh-R.

The inhibitory assays of PhTX on Glu-R and nACh-R were carried out as follows: (i) qGlu-R: inhibitory potency of neurally-evoked twitch contraction of locust skeletal muscle (ref. 14); (ii) vertebrate NMDA-R: displacement of the channel blocker [^{3}H] MK-801, rat brain cortex (ref. 16) ; (iii) nACh-R: displacement of the channel blocker [^{3}H] dodecahydrohistrionicotoxin, *Torpedo* electric organ (ref. 16). Selected results of the activity studies of PhTX on the qGlu-R and nACh-R are given in Figs. 2-4. The activities of the analogs are relative to PhTX-343 (IC_{50}: 2.3×10^{-5} M). The activity of PhTX against NMDA-R in general parallels its activity against the qGlu-R, but the overall activity range was smaller. Due to the inherent low potency of PhTX for the NMDA-R (IC_{50}: 4.4×10^{-5} M for PhTX-433) the binding results are not presented here. SAR of these analogs listed in Figs. 2-4 will be discussed later.

nACh-R AND PRELIMINARY PHOTOAFFINITY LABELING STUDIES

nACh-R is the major ion channel of the post-junctional membrane at the neuromuscular junction in vertebrates and in the electric organ of the ray *Torpedo*. This is probably the best understood receptor both pharmacologically and structurally, due to extensive studies especially by the groups of Changeux, Hucho, Karlin, Numa, and Unwin (ref. 18). It is a membrane bound 270 KDa glycoprotein with two external agonist binding sites and an internal ion channel, formed from the five subunits, α, α', β, γ, δ (Fig. 5)(ref. 18). Each subunit consists of four membrane-spanning regions, and the M2 helical region, believed to be α-helical, lines the inner pore. The acetylcholine binding site is located on the two α subunits halfway down the synaptic side. Close to the receptor in the cytoplasmic interior, there exists a 43 KDa protein. As depicted in Fig. 5, there exists a constriction in the gate which is blocked by the noncompetitive antagonist chlorpromazine (modified from ref. 18b).

At low concentrations (10^{-11} to 10^{-7} M), PhTX potentiates the activity of vertebrate CNS NMDA-R and KAIN-R, and of *Torpedo* nACh-R, thus suggesting the presence of more than one binding site (ref. 15, 19). As in Glu-R, at least two PhTX binding sites are thought to exist on nACh-R, as is evidenced by potentiation of [^{3}H]acetylcholine binding to electric organ receptor in the presence of low concentrations of PhTX (ref. 19). At high concentrations (0.1 to 1000 μM), PhTX-433 antagonizes

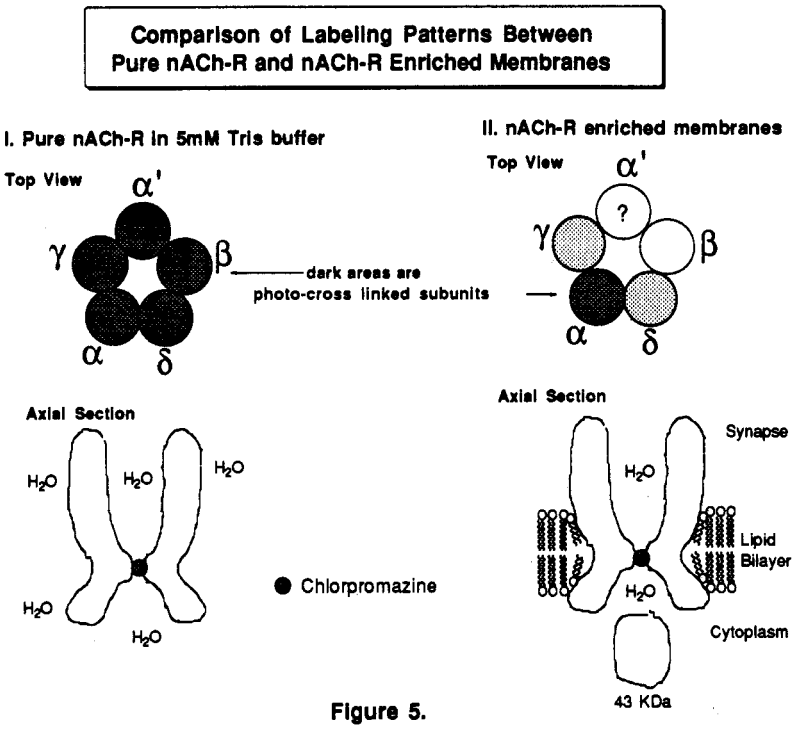

Figure 5.

the nACh-R as determined by patch clamp studies of frog muscle and cockroach ganglia, or biochemical methods (honeybee brain and *Torpedo* electric organ)(ref. 20). The activity of PhTX is *use-dependent*, namely, it results from open channel block of the activated nACh-R. PhTX also reversibly inhibits the response of *Xenopus* oocytes (frog eggs) injected with rat brain or chick brain mRNA encoding NMDA-R and non-NMDA-R (refs. 15, 19). Photoaffinity labeling studies performed with nACh-R using the radioactive N_3-Ph-$^{125}I_2$-PhTX-343-Lys (Fig. 6) depicted in Fig. 5 showed that without the 43 KDa protein, all five subunits became linked to the toxin. In contrast, when parallel experiments were performed with a receptor membrane still containing the 43 KDa protein, fewer subunits became radioactive (SDS PAGE)(ref. 21); this could suggest the unsymmetric disposition of PhTX by the 43 KDa protein with respect to nACh-R. Photolabeling studies using the same analog followed by trypsin cleavage and SDS PAGE/autoradiogram (including mass spectral measurements) showed that the peptides linked to the the PhTX analog had a MW of less than 2.5 KDa. Since all membrane-spanning peptides expected from trypsin cleavage have MW of >> 2.5 KDa, this demonstrates that the azidophenyl moiety is in the cytoplasmic region and not inside of the membrane spanning region. This leads to the picture shown in Fig. 6 in which the polyamine is extended into the internal ion channel from the cytoplasmic side.

MODE OF BINDING OF PhTX TO nACh-R

It is possible to rationalize the extensive SAR studies (Figs. 2-4) and preliminary photoaffinity binding studies by a model in which the polyamine chain inserts into the open channel from the cytoplasmic side. As depicted in Fig. 6 (modified after ref. 18d) the five M2 segments lining the gate are arranged in such a manner that six circles are present, consisting of two anionic (top and bottom), one hydrophobic (leucine) and three hydrophilic rings (ref. 18). With PhTX, it is possible to line the polyamine ammoniums against the hydrophilic circles and the hydrophobic regions of PhTX (III and IV)

outside the M2 pore. The following comments commonly apply to Glu-R as well as nACh-R, except when stated otherwise.

(i) Region I. The long polyamine chain is essential. The reason for small differences between PhTX-343 (relative activity 1.0), PhTX-433 (natural, 1.3) and PhTX-334 (1.5, not listed) against qGlu-R presumably arises from subtle differences in the alignment of the hydrophilic Ser, Thr, Glu and Gln groups in the three rings and the distances between the ammonium groups in the polyamine (Fig. 6). The increased potency in all three receptors shown by the butyl analog (Bu-433) extending from the very hydrophilic polyamine chain should be noted; the hydrophobic butyl group probably increases the affinity through hydrophobic binding to Leu-251 (?). Quaternization of amino groups drastically reduces the activity; it is likely that the bulk of the hydrophobic methyl groups hinders the hydrogen-bond stabilized links between, for example serine and the polyamine chain.

(ii) Region II. Extension of the polyamine chain enhances activity (cf. spider toxin structures). For example, extension by an Arg could extend the H-bond stabilization to Thr-260 (?).

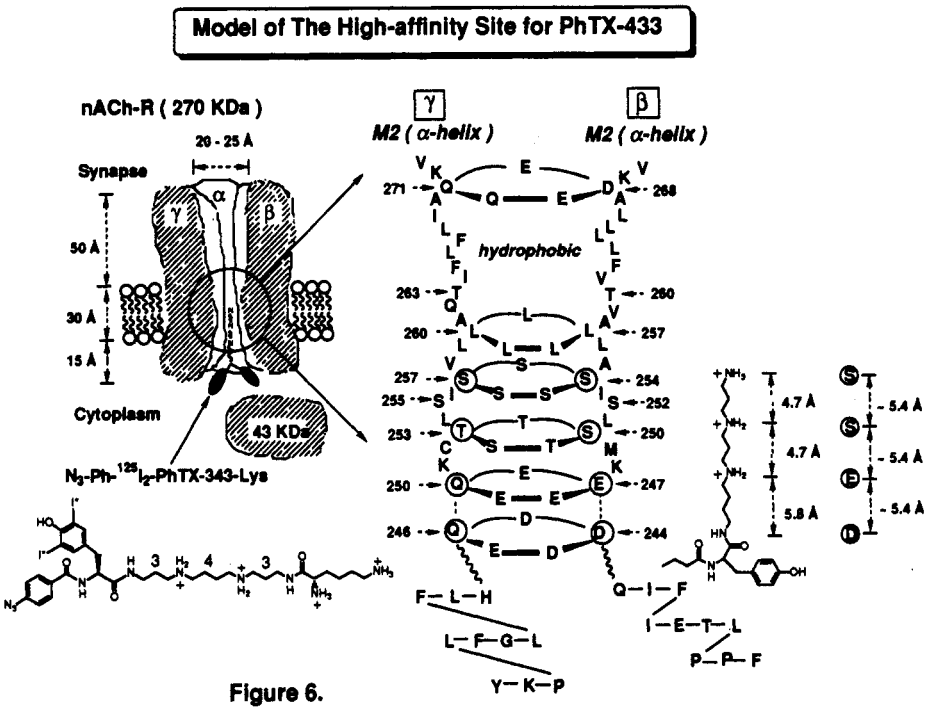

Figure 6.

(iii) Region III. Activity is enhanced by hydrophobic groups but is drastically reduced by hydrophilic groups as in (Asp). Regions III and IV probably interact with the hydrophobic amino acid moieties present on the cytoplasmic side of the channel. The high potencies of the azidophenyl analog (N_3Ph) and the cinnamate analog are encouraging since these aromatic residues can be utilized in photolabeling studies.

(iv) Region IV. Here some differences were noticed between qGlu-R and nACh-R. In the former case, a bulky anchoring group with moderate hydrophobicity appears to be necessary. The hydroxyl group on Tyr is not required; rather activity is increased in the Phe analog. The systematic activity enhancement accompanying halogenation of the aromatic nucleus is noteworthy. It is conceivable that this trend is related to the operation of a polarizability effect during binding. The enhanced activity in the iodinated analog is important since this not only leads to analogs with 4 to 9-fold enhanced affinity

through an iodination that can be performed at the last stage, but also to radiolabeled ^{125}I analogs (ref. 22). The activity with the nACh-R, unlike that with qGlu-R, is not affected by removal of the bulky aromatic moiety. Finally, the qGlu-R affinity is not dependent on the configuration of the Tyr group, the D-isomer being as active as the native L-isomer.

To study the interaction of PhTX with the ligand-gated ion channel receptors, doubly and triply modified analogs of PhTX were prepared containing photolabile moieties. For qGlu-R, the highest potency derivative was I_2-PhTX-343-Arg having two iodines in the Tyr moiety and region I extended by Arg; this resulted in a 33-fold increase in activity. The activity of PhTX analogs is reversible (refs. 1,3a). This has important biological implications since it is important for the wasp or spider to paralyze its prey for storage but not to kill it (ref. 23). Several classes of photosensitive analogs carrying affinity labels at different sites have been prepared based on the results of the structure activity studies; when these photosensitive analogs were applied to the locust leg nerve muscle preparation at concentrations of 10^{-9} M, a reversible block of excitatory postsynaptic currents was seen. However, irradiation with 270 nm light after application led to an irreversible block. These results demonstrated that the photoaffinity labeled toxins were properly delivered to the binding and that they were functioning as expected, namely, to become covalently linked to the binding site upon irradiation (ref. 24).

When applied externally to locust muscle at high concentrations (ca. > 10^{-6} M), PhTX was shown to be a *use (agonist)-dependent* antagonist of qGlu-R by single channel studies (refs. 15, 19). However, microinjection of PhTX-343 *into* locust muscle fibres led to *use-independent* antagonism (ref. 20). A rationalization for this is that the PhTX binding site may be located in the cytoplasmic domain of the receptor, so that internal injection leads to direct binding to the receptor and hence immediate antagonism. In contrast, when the toxin is applied externally to the locust muscle, the toxin must first pass through the open ion channel to reach its binding site and thus is *use-dependent*. The trends are all consistent with the mode of binding proposed in Fig. 6. Further studies are ongoing using more extensively modified analogs as well as photolabel sequencing coupled with solid phase chromatography and tandem MS/MS.

Acknowledgments

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