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The biological activities of five azido N-substituted phthalimides: potential photoaffinity reagents for gibberellin receptors[1]

Jeffrey C. Suttle[2], J.F. Hultstrand & F.S. Tanaka[3]
Biosciences Research Laboratory, USDA, Agricultural Research Service, P.O. Box 5674, State University Station, Fargo, ND 58105, USA

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Abstract

The biological activities of five azido derivatives of the synthetic GA-like bioregulator 94,377 (1-[3-chlorophthalimido]cyclohexanecarboximide) were examined in a range of gibberellin-sensitive assays including: barley half-seed α-amylase secretion, *Rumex* chlorophyll retention, d_5 maize leaf-sheath elongation, lettuce hypocotyl elongation and cucumber hypocotyl elongation. The five azido derivatives tested possessed an N-substituted phthalimide structure but differed in the placement of the azido moiety. An acyl-azido derivative was devoid of biological activity in all assays examined. Of the four remaining aryl-azido derivatives, three exhibited significant biological activity. The biological activity was both compound and species-dependent; a given azido derivative being highly active in one assay (species) but inactive in another. None of the aryl-azides promoted hypocotyl growth in light-grown cucumber seedlings when tested at 100 μM. However, two of the derivatives that were highly active in other assay systems were capable of displacing $[^3H]GA_4$ bound to a soluble binding protein prepared from cucumber seedlings when tested at high concentrations. These results indicate that certain aryl-azido derivatives of 94,377 may be useful in purifying GA binding proteins from responsive tissues and should facilitate the molecular modelling of the actual ligand binding pocket of GA receptors or other GA binding proteins.

Introduction

Progress in the area of plant hormone receptor biochemistry has lagged considerably behind advances in other areas of hormone physiology. Several factors have contributed to this situation including: 1) the limited *in-vitro* detectability of saturable hormone binding in plant tissue homogenates, 2) the difficulty of discerning physiologically relevant binding from artifactual associations, and 3) the *in-vitro* instability of hormone binding sites in plant tissue extracts. These difficulties have necessitated novel approaches including the use of immunological (anti-idiotype) techniques and photo-reactive hormone derivatives (photoaffinity labeling).

The preparation of potential photoaffinity ligands generally involves the introduction of an azido (typically an aryl-azido) moiety into the hormone of interest [1]. A critical aspect of this approach concerns the position of the azido function on the ligand. The introduction of the azido moiety must not interfere with ligand binding. However, the azido must be in a position proximal

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[2] Address for correspondence: Dr. Jeffrey C. Suttle, USDA-ARS Northern Crop Science Laboratory, P.O. Box 5677, State University Station, Fargo, ND 58105–5677.

[3] Present address: Dr. Fred S. Tanaka, BASF Corporation, Agricultural Research Center, POB 13528, Research Triangle Park, NC 27709-3528.

to the actual binding pocket to facilitate covalent linkage upon irradiation. The second of these criteria is difficult to predict, but the retention of biological activity serves as an imperfect index for the first requirement. Azido-containing, photoaffinity analogues of the cytokinins [15], phenoxyauxins [9], phytotoxins such as fusiccoccin [10], phytotropins [20] and IAA [6] have been prepared and used with varying degrees of success [11].

The fundamental processes that underlie gibberellin (GA) action remain unknown. Saturable *in-vitro* binding of radiolabeled GAs has only been detected in a limited number of tissue homogenates, including cucumber and pea [14]. In both cucumber and pea, the saturable binding sites appear to be soluble proteins [14]. Although only limited data have been presented, a particulate GA binding site(s) has been reported in potato homogenates [8]. Based on indirect evidence, a cell-surface (plasmalemma) location for GA binding has been inferred in wild oat protoplasts [4]. In no case has a reasonable degree of purification of these binding sites been achieved.

Previous studies in this laboratory have demonstrated that a group of synthetic bioregulators, the N-substituted phthalimides (NSP), are potent GA-like agonists in most tissues examined [16, 18]. Further, we have shown that bioactive members of this group competitively displace [^{3}H]GA_4 from soluble binding sites present in cucumber homogenates [21]. As a class, the chemistry of the NSPs is more amenable to modification that would be necessary for the preparation of an irreversible, photoaffinity GA-like ligand. Based on structural information gained from earlier studies, an initial group of five azido-NSPs has been synthesized [19].

In this report, the biological activities of these five azido-NSPs are described and the potential utility of these compounds for use as photoaffinity ligands for GA receptor(s) is discussed. Portions of this research have been presented previously in abstract form [17].

Materials and methods

Chemicals. Technical-grade (> 95% purity) 94,377 (Figure 1) was provided by American Cyanamid Co. (Princeton, N.J., U.S.A.). GA_3 was purchased from Sigma Chemical Co. GA_4 was provided by Abbott Laboratories and AMO-1618 was purchased from Calbiochem-Behring Corp. [^{3}H]GA_4 (33 Ci/mmole) was purchased from Amersham Corp. The synthesis of the azido-N-substituted phthalimides has been described [19]. All other reagents used were of the highest grade commercially available. Concentrated stock solutions of all growth regulators were prepared in acetone and were protected from light.

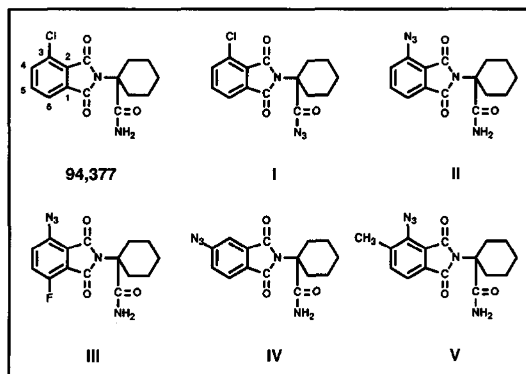

Fig. 1. The chemical structures of the N-substituted phthalimides used in these studies. The structure of the parent compound 94,377 is also shown together with the accepted numbering system for the phthalimide moiety.

Barley half-seed bioassay. The determination of the ability of the test compounds to stimulate the secretion of α-amylase from de-embryonated barley half-seeds was performed as described by Jones and Varner [7]. The barley seed used in these experiments was from the 1979 harvest and was obtained from the Agronomy Club of Washington State University, Pullman, WA, USA. α Amylase activity is expressed as total $\Delta OD_{620}.minute^{-1}$.

Rumex chlorophyll retention assay. Discs (6 mm, dia.) were excised from fully expanded leaves of *Rumex crispus* L. growing locally in a field. Treatment solutions were prepared in glass-distilled water. Leaf discs (5/replication) were incubated in glass 5 cm (dia.) petri dishes containing 3 ml of solution for 4 days in the dark (26 ± 1 °C). The leaf discs were then blotted dry and homogenized in 80% (v/v) aqueous acetone. After clarification by centrifugation for 10 min at 10,000 × g, chlorophyll

content was estimated spectrophotometrically (A_{665}).

Dwarf (d_5) maize assay. Seeds of the d_5 mutant of corn (*Zea mays* L.) were surface-sterilized using a 1 : 5 dilution of commercial bleach and were germinated in vertically oriented cylinders of moist paper towelling. After 6 days of growth in a chamber (16 h photoperiod, 250 $\mu E\, m^{-2} sec^{-1}$ provided by cool-white fluorescent and incandescent bulbs, 25/23 °C day/night temperature), the seedlings were transferred to foil-wrapped glass jars (3/jar; ca. 250 ml volume) containing the test compounds dissolved in 1/4 strength nutrient solution [3]. The seedlings were returned to the chamber for a further 7 days, at which time the cumulative length of leaf sheaths 1 plus 2 were determined to the nearest mm.

Lettuce hypocotyl assay. Achenes of lettuce (*Lactuca sativa* L. cv. Grand Rapids) were germinated in the dark on moist filter paper at 26 ± 1 °C for 18 hours. The compounds to be tested were dried on discs of filter paper in 20 ml beakers in the dark. After drying, the filter paper was wetted with 1 ml of a solution of 100 μM AMO-1618 prepared in glass-distilled water. The sprouted achenes (Radical length: 2–4 mm; 7/beaker) were added to each beaker and incubated in the dark (26 ± 1 °C). Final hypocotyl length (to the nearest mm) of 5 representative seedlings/beaker was determined after 3 days of growth.

Sunflower and soybean epicotyl assays. Seeds of either sunflower (*Helianthus annuus* L. cv. NK265) or soybean (*Glycine max* L. cv. Wilkin) were surface-sterilized using a 1 : 5 (v/v) dilution of commercial bleach and were germinated in vertically oriented cylinders of moist paper towelling. After 5 (sunflower) or 6 (soybean) days of growth in a chamber (16 h photoperiod; 250 $\mu E\, m^{-2} sec^{-1}$ provided by cool-white fluorescent and incandescent bulbs; 25/23 °C day/night temperature), the seedlings were transferred to foil-wrapped glass jars (3/jar; ca. 250 ml volume) containing 1/3 (sunflower) or 1/4 (soybean) strength nutrient solution (3) and returned to the growth chamber. After 24 hours, the nutrient solution was replaced with one of identical strength containing the test compounds. Final epicotyl (first internode) length was determined to the nearest mm after 8 (sunflower) or 7 (soybean) days of treatment.

Cucumber hypocotyl and in-vitro binding assays. Seeds of cucumber (*Cucumis sativus* L. cv. National Pickling) were germinated between moistened paper towels at 26 ± 1 °C in a dark chamber for 2 days. Seedlings were then transferred to glass jars containing 1% (w/v) agar. The test compounds were dissolved in the agar by adding an acetone stock solution to the agar solution at 45 °C with rapid stirring. The seedlings were then placed in a growth chamber (16 h photoperiod provided by fluorescent and incandescent bulbs; 100 $\mu E\, m^{-2} sec^{-1}$; 25/23 °C day/night temperature) for 4 days. Final hypocotyl length of 10 representative seedlings was then determined to the nearest mm.

The GA-binding protein from cucumber seedlings was prepared as described previously [21] and was stored at −70 °C. The *in-vitro* [^{3}H]GA_4 binding assay was performed as described earlier [21]. Briefly, compounds to be tested were dissolved in acetone, added to glass vials and the acetone was allowed to evaporate at room temperature in the dark. The GA-binding protein preparation was thawed, diluted to a final protein concentration of 2 mg/mL with binding buffer (20 mM Bis Tris, 50 mM $MgCl_2$, 1 mM EDTA, pH 6.5) and added to a vial containing [^{3}H]GA_4 (33 Ci/mmole; final concentration: 10 nM). Aliquots of this mixture (0.5 mg protein) were then added to the vials containing the test compounds. Binding was allowed to proceed for 90 min in the dark at 4 °C. Separation of bound from free [^{3}H]GA_4 was accomplished using the DEAE filter disc technique described earlier [21]. Non-saturable binding was defined using 1 μM unlabeled GA_4.

Results

Previous studies with various NSPs [21] provided limited initial information concerning structural requirements for binding and were used in the design of the azido derivatives synthesized (Figure 1). Azido I (an acyl azido) was synthesized in accordance with observed requirements for substitution at the carboxyl moiety. Acyl-azido derivatives of estrogens have been used as probes of the estrogen receptor [12]. The aryl azides II, IV, V

Table 1. Biological activities of GA_3, GA_4, 94,377 and azido NSPs in various GA-responsive assay systems. Values presented are the mean (SE). Details for each assay are provided in the Materials and methods section.

Compound	Barley α-amylase		Rumex leaf senescence		d_5 leaf sheath length		Lettuce hypocotyl length		Sunflower epicotyl length		Soybean epicotyl length	
	Dose[a]	ΔOD_{620}	Dose[a]	OD_{665}	Dose[a]	cm	Dose[b]	mm	Dose[a]	cm	Dose[a]	cm
Control	-	6.11 (0.56)[c]	-	0.063 (0.002)[c]	-	4.0 (0.2)[d]	-	13 (1)[e]	-	7.1 (0.2)[d]	-	11.3 (0.4)[d]
GA_3	-	nt[f]	100	0.559 (0.013)	100	16.7 (1.4)	100	20 (1)	100	11.1 (0.5)	100	22.1 (0.8)
GA_4	1	32.19 (1.57)	-	nt	-	nt	-	nt	-	nt	-	nt
94,377	100	28.17 (0.42)	100	0.496 (0.022)	100	14.9 (0.8)	100	20 (1)	100	8.5 (0.3)	100	19.2 (0.7)
I	30	9.46 (0.54)	100	0.139 (0.026)	100	4.3 (0.1)	100	14 (1)	100	7.3 (0.1)	-	nt
II	100	20.20 (1.30)	100	0.193 (0.033)	100	6.0 (0.3)	100	18 (1)	100	9.0 (0.2)	100	13.8 (0.3)
III	100	18.04 (0.5)	100	0.184 (0.016)	100	4.8 (0.3)	100	13 (1)	100	6.8 (0.4)	10	11.5 (0.7)
IV	100	16.65 (1.69)	100	0.138 (0.013)	100	5.9 (0.2)	100	10 (1)	100	7.5 (0.4)	100	16.0 (0.4)
V	100	18.70 (1.16)	100	0.289 (0.011)	100	7.6 (0.3)	100	17 (1)	100	9.0 (0.4)	100	13.7 (0.6)

[a] μM.
[b] μg/beaker.
[c] n = 3.
[d] n = 6.
[e] n = 5.
[f] nt = not tested.

were designed such that an azido moiety was substituted for a halogen in a bio-active NSP (compare II with 94,377). A fluorine was introduced *para* to the azido function in II (yielding III) in order to generate a more reactive photo-induced intermediate [13].

All five azido-NSP derivatives were evaluated in a range of GA-sensitive bioassays of varying degrees of specificity. The biological activity in each assay was compared to that elicited by a gibberellin (GA_3 or GA_4) and the most potent NSP identified to date (94,377). It should be noted that most of the assays employed were modified considerably in order to accommodate the photo-instability of the azido derivatives. In many instances these modifications attenuated the overall responsiveness of the assay. Nevertheless, growth promotion by active compounds was readily detectable.

Exposure of barley half-seeds to GA_4 resulted in an expected increase in α-amylase secretion (Table 1; column 1). The GA_4-induced increase was evident at a concentration of 0.01 μM (lower concentrations were not tested) and increased with increasing GA_4 concentration to 1 μM. The non-photolysable NSP 94,377 was considerably less potent than GA_4 but ultimately induced the maximum response when applied at 100 μM. With the exception of azido I, the azidos III, IV, V elicited a detectable but sub-maximal increase in α-amylase secretion. Azido II appeared to be the most potent in this assay and its action was studied in more detail (Figure 2). A detectable increase in α-amylase secretion was observed at a concentration of 1 μM. This stimulation increased in a log-linear fashion as the concentration of II was increased from 1–100 μM. The bioactivity of II observed at 100 μM was equivalent to a saturating level of GA_4 (1 μM). Higher concentrations were not tested due to solubility limitations.

The dark-induced loss of chlorophyll in *Rumex* leaf discs was significantly retarded by both GA_3 and 94,377 (Table 1; column 2). To varying degrees, all azido-NSPs elicited a similar retarda-

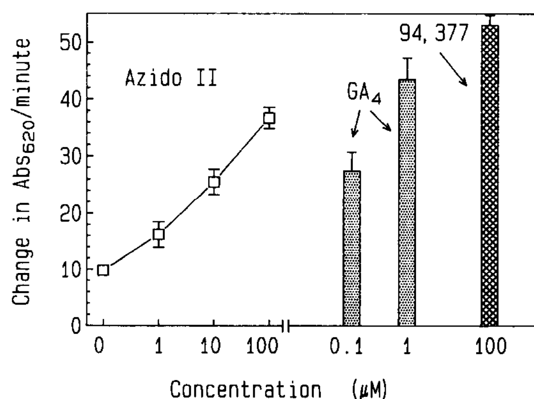

Fig. 2. Dose-response study of the bioactivity of azido II in the barley half-seed α-amylase secretion assay. The responses of the assay to 100 μM GA_4 are also indicated.

tion. Azidos I and IV were only marginally effective while II and III elicited a moderate response. Azido V was the most active member of this group but still elicited only a partial response (i.e. 52% of the response to GA_3). In previous studies [18], the threshold response to GA_3 and 94,377 in this assay was 0.03 and 3 μM respectively. The threshold response to azidos II and V was found to be 3 and 1 μM respectively.

Application of both GA_3 and 94,377 to dwarf (d_5) corn seedlings by root-feeding resulted in a pronounced stimulation of leaf sheath elongation (Table 1; column 3). Azidos I and III were inactive in this system, while II, IV and V elicited a moderate stimulation. Previous research has shown that, when applied by root-feeding, the threshold response to GA_3 and 94,377 occurred at 0.01 μM and 1 μM, respectively [16]. Azido V was the most potent derivative in this assay and its effective threshold concentration was 1 μM.

Preliminary studies had shown that none of the azido-NSPs exhibited biological activity in the standard lettuce hypocotyl bioassay which is conducted in the light. This inactivity presumably reflected the rapid photo-decomposition of the azido derivatives on the filter discs when illuminated. By including a growth retardant such as AMO-1618 in the wetting medium, it was found that this assay could be conducted entirely in the dark. This modification reduced the sensitivity of the assay to both GA_3 and 94,377 but permitted a direct comparison of the activities of all compounds simultaneously. Both GA_3 and 94,377 (100 μg/beaker) elicited a pronounced stimulation of lettuce hypocotyl elongation in dark-grown seedlings (Table 1; column 4). A threshold dose for bioactivity of 1 μg/beaker was found for both GA_3 and 94,377. Azidos I, III and IV were inactive in this assay when tested with at 100 μg/beaker (Table 1) or at 1 or 10 μg/beaker. Compounds II and V were only slightly less active than GA_3 or 94,377 in this assay. The threshold dose for bioactivity of either of these two azido derivatives was 10 μg/beaker.

The ability of these derivatives to stimulate stem elongation in two other non-standard but GA-responsive systems was also evaluated. Both GA_3 and, to a lesser extent, 94,377 stimulated epicotyl elongation in light-grown sunflower and soybean seedlings (Table 1; columns 5 and 6, respectively).

Table 2. Effects of GA_4, 94,377 and azido-NSPs on hypocotyl elongation and saturable [^{3}H]GA_4 binding in cucumber (*Cucumis sativus* L.) seedlings and homogenates. For the elongation studies, compounds (100 μM) were fed continuously via the transpiration stream. The binding displacement studies were conducted using the soluble GA-binding protein(s) present in cucumber homogenates. [^{3}H]GA_4 binding was assayed at two concentrations of competitor (50 & 500 μM). Non-specific binding was defined using 1 μM unlabeled GA_4 and was found to be between 25–30% of the total radioactivity trapped on the filters.

Compound	Hypocotyl Length (cm)[1]	[^{3}H]GA_4 Specifically Bound (pmol/mg protein)[2]	
		[Competitor, μM]	
		50	500
None	2.5 ± 0.1	2.62 ± 0.19	2.61 ± 0.16
GA_4	9.7 ± 0.2	-	-
94,377	7.4 ± 0.2	0.93 ± 0.11	0.36 ± 0.16
I	2.7 ± 0.1	2.53 ± 0.99	2.81 ± 0.16
II	2.8 ± 0.1	2.52 ± 0.21	1.64 ± 0.02
III	2.8 ± 0.1	ND[3]	ND[3]
IV	2.7 ± 0.1	2.69 ± 0.68	2.21 ± 0.09
V	2.8 ± 0.1	2.21 ± 0.15	1.25 ± 0.13

[1] Mean ± SE (n = 10).
[2] Mean ± SE (n = 3).
[3] Not determined.

Azido I was inactive in the sunflower assay and, due to limited availability, was not tested in soybean seedlings. Compound III was also inactive in both seedlings and actually exhibited phytotoxicity in soybean seedlings at 100 μM. Compound IV was inactive in sunflower seedlings but was very active in the soybean assay. Azidos II and V were equally potent in both assays.

In addition to these bioassays, the efficacy of the azido-NSPs was examined in other systems as well. All five azido-NSPs were inactive when root-fed at 100 μM to dwarf pea (cv. Little Marvel) and dwarf Tan-ginbozu rice seedlings. GA_3 exhibited activity in both assay systems while 94,377 was active only in the dwarf rice assay.

Both *in-vivo* biological activity and *in-vitro* saturable GA binding can be demonstrated using cucumber seedlings. Previous studies [21] have examined the effects of a range of NSP derivatives *in-vivo* and *in-vitro* in cucumber seedlings. For this reason, the *in-vivo* and *in-vitro* activities of the azido-NSPs were examined in greater detail in this system (Table 2). When root-fed at 100 μM, both GA_4 and 94,377 elicited considerable hypocotyl elongation in these seedlings. In contrast, all of the

azido-NSPs were inactive *in-vivo*. As expected, saturable [^{3}H]GA_4 binding activity was present in 100 k x g supernatants prepared from cucumber hypocotyl homogenates. Saturable [^{3}H]GA_4 binding was reduced in the presence of either 50 or 500 μM 94,377. Previously [21], an IC_{50} value of 58 μM for 94,377 was found in this binding assay. With the possible exception of azido V, none of the azido-NSPs displaced [^{3}H]GA_4 from these binding sites when tested at 50 μM. At a concentration of 500 μM, azido-NSPs I and IV were still without effect while II and V exhibited significant displacing abilities. Due to unavailability at this point, azido III was not tested *in-vitro*.

Discussion

To date, no successful purification of the GA receptor(s) has been reported. In fact, saturable *in-vitro* binding of radiolabeled GAs has only been detected in extracts prepared from a limited number of tissues [14]. Even in these cases, the receptors have proven recalcitrant to further purification. The underlying bases for the limited detectability and *in-vitro* instability are unknown.

As an alternative, a series of photolabile, potentially irreversible ligands for the GA receptor(s) has been synthesized [19]. The molecular design of these potential ligands was based on bioactive members of the N-substituted phthalimides, a class of synthetic GA-like agonists [21]. Aryl azide derivatives have proven extremely useful in the detection and purification of both vertebrate and plant hormone binding proteins [1, 11]. The azido-NSPs described in this paper have proven to be stable when handled under subdued lighting and are readily photolyzed when exposed to direct UV illumination [17].

The results presented in Table 1 demonstrate that, depending on the assay used, four of the five azidos synthesized (compounds II–V) exhibit moderate GA-like activity. The acyl-azide (I) was the least active member of the group, displaying GA-like activity in only two assays (α-amylase secretion and *Rumex* chlorophyll retention). These results were not unexpected as previous studies [21] had indicated that the nature of the substituent at this position was critical with regard to both biological activity and *in-vitro* binding capacity. Compound III also exhibited limited biological activity. Although more potent in eliciting a GA-like response, it too was active only in the α-amylase secretion and *Rumex* chlorophyll retention assays. Interestingly, III exhibited no promotive activity in assays requiring tissue growth (i.e., the d_5, lettuce, sunflower and soybean assays) and actually exhibited phytotoxicity (inhibition of root elongation) at high concentrations.

Of the azidos synthesized, compounds II, IV and V elicited the greatest degree of GA-like activity. In general, II and V exhibited the greatest response in the widest range of assays. However, tissue specificity was again manifested. For example, IV was among the most active in soybean seedlings (Table 1; column 6) but was inactive in the lettuce hypocotyl and sunflower epicotyl assays (Table 1; columns 4 and 5). The biological activity of II and V was anticipated as these compounds are azido analogs of highly active halogen-containing NSPs (compare 94,377 with II, and see [21]). The biochemical bases for the tissue selectivity observed in these studies are unknown. Previous studies have demonstrated that 94,377 is rapidly metabolized *in-vivo* and is poorly translocated, exhibiting only acropetal movement [16]. Thus, differences in metabolic stability and/or translocatability as related to chemical structure may explain these observations. It should be noted that the observed bioactivity of these azido NSP derivatives in the elongation studies conducted in the light probably underestimates the actual potency of the compounds. The protracted treatment time required for these assays undoubtedly resulted in significant photolytic degradation of these compounds in spite of the precautions taken.

The apparent inactivity of all the azido-NSPs in the cucumber hypocotyl assay was unexpected (Table 2). A variety of treatment protocols was employed (including seed treatment, direct application to the hypocotyl, etc.), in an attempt to circumvent any limitations to bioactivity resulting from limited movement, metabolism, and/or photolysis. In no case was bioactivity observed. *In-vitro* binding studies (Table 2) indicated that only the azidos II and V exhibited any affinity for the soluble GA-binding protein present in cucumber homogenates. However, even for these two compounds, the binding affinity (as judged by competition assays) was limited; the IC_{50} concentration

being greater than 500 μM for both. This limited affinity for the presumed receptor would explain the failure to observe *in-vivo* biological activity when tested at 100 μM (near the limit of solubility in nutrient medium).

The near total loss of binding affinity when replacing a halogen moiety on a bioactive NSP (94,377) with an azido group (II) is puzzling. This type of substitution has proven successful for a wide-range of ligands of varied molecular weight [1]. In the case of plant growth regulators, azido analogs of halogen-containing derivatives of 6-benzyladenine, indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid and N-1-naphthylphthalamic acid exhibited significant bioactivity both *in-vivo* and in binding assays [6, 9, 15, 20]. In some cases, substitution of an azido moiety for a halogen had no effect on activity [15] while in other cases a reduction in both *in-vivo* and *in-vitro* activity was noted [9, 20]. Previously, it was observed that analogs of the highly active NSP 94,377 with a methyl, iodo or amino substituent at position 3 of the NSP phenyl ring (Figure 1) were also highly active [21]. The present results indicate that the nature of the substituent at this position is critical and deserves more study.

The gibberellin molecule has been chemically modified in an attempt to generate a photo-reactive ligand [2, 5]. In these studies, an aryl-azide was introduced via a thio-propyl linker attached to the 17 position of GA_4. In an aleurone protoplast α-amylase assay, the azido-GA_4 derivative was approximately one hundred fold less active than GA_4 itself [5]. Maximum stimulation was observed at 10 μM azido-GA_4 as compared to 0.01 μM GA_4. This degree of bioactivity compares well with that of compound II in this study which elicited full activity between 10 and 100 μM (Figure 2).

In most cases, the azido derivatives were partial agonists; exhibiting sub-optimal activity at the highest doses tested. Often, partial agonists have proven to be antagonists when tested at lower doses. This possibility was explored using azido II in conjunction with GA_3 application in the lettuce hypocotyl, cucumber hypocotyl and d_5 leaf sheath assays. No evidence for antagonism was noted.

The low affinity of these azido derivatives for the cucumber GA binding protein(s) coupled with their lack of biological activity *in vivo*, indicates that they would be of limited use in the detection and purification of the binding sites in this tissue. However, azidos II and V exhibited significant *in vivo* biological activity in several of the assay systems studied (Table 1). In particular, the bio-activity of II approached that of the parent NSP 94,377 in the barley α-amylase assay and II and V were as active as 94,377 in sunflower seedlings. Although the activity of all the NSPs studied to date is considerably less than that of GA_3 or GA_4, they do exhibit apparent affinities for soluble GA binding sites in the micro-molar range [21]. Other photo-affinity ligands with similar binding affinities have been successfully employed to purify binding sites in other systems [1]. Thus, suitably radio-labeled derivatives of azidos II, IV or V may be of use in the characterization of GA binding proteins in tissues such as barley aleurone, sunflower and soybean seedlings where the observed bio-activity presumably reflects a significant affinity for the GA receptor in these tissues. Radiolabeling of these compounds could be achieved by catalytic tritiation of a nitro-containing synthetic intermediate [19]. In addition, these azidos derivatives should facilitate the molecular characterization of the ligand binding pocket of GA binding proteins isolated by other means. Further improvement of both biological activity and binding affinity may be achieved by the systematic exploration of the activity of derivatives with alterations in the cyclohexane moiety of this class of bioregulators.

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