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Studies on the Profile of Immunostimulant Activities of Modified Iridoid Glycosides[1]

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Abstract—Immunostimulant activity profile of modified iridoid glycosides prepared from loganin (**1**), ketologanin (**2**) and arbortristoside A (**3**) have been studied and some structure–activity relationships have been obtained.

Introduction

Iridoid glycosides, a class of naturally occurring compounds containing cyclopentan (C) pyran as the aglycon part and β-D-glucopyranose as the sugar residue, are known to be associated with immunostimulant and hepatoprotective activities.[1–10] However, no precise study has ever been made to understand the impact of structural modification on the immunostimulant profile of these glycosides. For generating such an information various types of changes were made in the β-D-glucopyranose and aglycon residues as shown in Figure 1 (Tables 1–5) and the resulting compounds were subjected to immunological evaluation. Compounds bearing structural changes in the sugar residue were prepared more in numbers because carbohydrate moieties are known to carry specific immunological messages as antigens or haptens.[11,12] The details of the study are presented here.

Chemistry

The starting materials namely loganin (**1**) and arbortristoside A (**3**) were isolated from plants *Strychnos nux vomica* (fruits) and *Nyctanthus arbortristis* (seeds), respectively. The 7-ketologanin (**2**) was prepared from loganin (**1**) by Jones oxidation[13] and all other modified iridoid glycosides (**4**–**25**) were prepared by the methods reported earlier by our group.[14–16] The aglucon (**26**) of Loganin (**1**) was prepared by the reaction of **1** with β-glucosidase.[13]

Immunological evaluation

In the present study, the main objective is to identify an agent which can elicit the desired immune responses[11] of the host for providing immune support during chemotherapy. Since this immune support is required for parasite clearance, monitoring of T-cell response acquires importance. Keeping this as the goal, following parameters were selected for the evaluation of these compounds.

1. Cell mediated immune responses (CMI)
   (a) Non specific macrophage migration index (MMI, in vivo)
   (b) Lymphocyte transformation index (TI, in vitro)
2. Humoral immune response (HI, in vivo)
   (c) Antigen specific haemagglutination titre and
   (d) Plaque forming cells (PFC)/10^6 spleen cells.

Animals and treatment

Male Swiss mice (20–22 g) were obtained from the animal house of the Central Drug Research Institute (CDRI), India. The animals were kept on a standard pelleted diet (Lipton India, Chandigarh) and water ad

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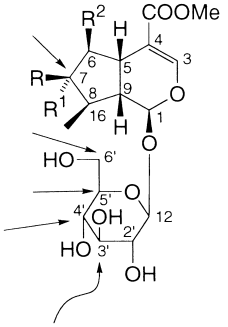


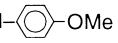
1. R = OH, R^1 = H, R^2 = H
   (Loganin)
2. R,R^1 = O, R^2 = H
   (Ketologanin)
3. R = OCOCH=CH–  –OMe
   R^1 = H, R^2 = OH
   (Arbortristoside A)

Figure 1. Loci of modifications at sugar residue and aglycon part.

libitum. Mice were divided into groups of six each and were injected with 4 mg/kg dose of the test compound intraperitoneally (i.p.) on days −7 and −3 (one group for one compound). The animals of one group which served as controls were given saline only. On day 0 the mice were studied for specific and non-specific immune responses.

Non-specific (antigen independent) immune response

Macrophage migration index (MMI)

Three animals of each group were used for measuring MMI according to the method of Saxena et al.[17] Briefly peritoneal exudate cells (PEC) were collected from treated and untreated animals, packed in microhaematocrit capillary and cultured overnight at 37 °C in a migration chamber filled with RPMI-1640 medium containing 10% fetal calf serum. The area of PEC migration from the capillaries into the migration chamber was marked on Whatman No. 1 filter paper using camera Lucida and weighed. Migration area of PEC from untreated normal mice was also marked and similarly weighed. The ratio of the area of migration of cells from treated animals (A_1) to that of untreated animals (A_2) has been expressed as macrophage migration index.

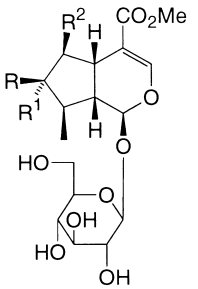
$$\mathrm{MMI} = \frac{A_1}{A_2}$$

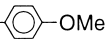
Specific immune response

Remaining three mice of test compounds treated and untreated (control) groups were injected each with 1×10^8 sheep red blood cells (SRBC) i.p. on day 0. After four days, blood was collected from the retroorbital plexus for the determination of HA titre and spleen was taken out for PFC assay.

Haemagglutinating antibody (HA) titre. HA titre was determined in the sera obtained from the immunized animals by the microtitre plate method.[18] In brief, 50 μL aliquots of twofold serial dilutions (2-4096) in phosphate buffered saline (PBS) were prepared in one

Table 1. Immunodulatory activities of loganin (**1**), ketologanin (**2**) and arbortristoside A (**3**)



Compd no. and structure	HA titre	Plaque forming cells	Macrophage migration index
1. R = OH, R^1 = R^2 = H (Loganin)	1536 ± 229	190 ± 25	2.61 ± 0.10
	(469 ± 43)	(110 ± 10)	
2. R,R^1 = O, R^2 = H (7-Ketologanin)	512 ± 0	110 ± 12	1.20 ± 0.02
	(512)	(107 ± 15)	
3. R = OCOCH = CH–  –OMe R^1 = H, R^2 = OH (Arbortistoside A)[10]	1365 ± 147	356 ± 40	2.6 ± 0.76
	(469 ± 38)	(90 ± 7)	

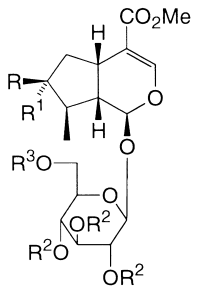
Values are mean ± standard deviation (s.d.) of six animals in each case.
Data in parentheses denote the control values.

row of a 96-well microtitre plate. Twenty-five micro litres of 1% fresh SRBC suspension in PBS was dispensed into each well and mixed thoroughly. The plate was incubated at room temperature for 2 h and examined for agglutination. The reciprocal of the highest dilution of the test giving visible agglutination has been expressed as HA titre.

Plaque forming cell (PFC) assay. The assay was performed according to Jerne and Nordin.[19] The spleen cells were separated in RPMI-1640 medium, washed twice and suspended in the same medium to a concentration of 1×10^7 cells/mL. Glass Petri dishes (6.0 cm diameter) were layered with 1.2% agarose in 0.15 M NaCl to form the bottom layer. Then a mixture comprised of 2 mL agarose (0.6%) in RPMI-1640 medium (40 °C), 0.1 mL suspension of 20% SRBC and 1×10^6 spleen cells in a volume of 0.1 mL was poured over the bottom layer of agarose and the petridishes were incubated at 37 °C for 90 min. Two mL of 1:10 diluted fresh guinea pig serum was added in each Petri dish as a source of complement and the plate was reincubated for 45 min. The number of plaques were counted immediately. The values have been expressed as counts per 10^6 spleen cells.

Two similar type of experiments were conducted for each of the compounds studied.

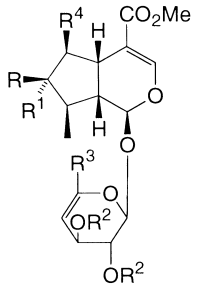
Table 2. Immunomodulatory activities of loganin analogues



Compd no. and structure	HA titre	Plaque forming cells	Macrophage migration index
4. R,R^1 = O, R^2 = R^3 = Ac	512 ± 0 (1.0)	120 ± 14 (1.1)	1.2 ± 0.03
5. R = OAc, R^1 = H, R^2 = R^3 = Ac	1024 ± 0 (2)	205 ± 0 (1.86)	1.73 ± 0.25
6. R = OAc, R^1 = H, R^2 = Ac, R^3 = CO–C$_6$H$_5$	512 ± 0 (1.0)	115 ± 10 (1.0)	1.62 ± 0.12
7. R = OAc, R^1 = H, R^2 = Ac, R^3 = CO–C$_6$H$_3$(OMe)$_2$	512 ± 0 (1.0)	115 ± 12 (1.0)	1.70 ± 0.02
8. R = OAc, R^1 = H, R^2 = Ac, R^3 = CO–C$_6$H$_2$(OMe)$_3$	1024 ± 0 (2.0)	115 ± 10 (1.0)	1.62 ± 0.06
9. R = OAc, R^1 = H, R^2 = Ac, R^3 = COCH=CH–C$_6$H$_4$–OMe	512 ± 0 (1.0)	125 ± 17 (1.2)	1.27 ± 0.02
10. R = R^1 = O, R^2 = H, R^3 = C(Ph)$_3$	819 ± 125 (1.6)	130 ± 25 (1.2)	1.81 ± 0.64
11. R = R^1 = O, R^2 = Ac, R^3 = C(Ph)$_3$	1706 ± 528 (3.2)	210 ± 25 (1.19)	2.15 ± 0.21
12. R,R^1 = O, R^2 = Ac, R^3 = H	597 ± 195 (1.1)	115 ± 10 (1.0)	1.88 ± 0.17
Control	512 ± 0	110 ± 10	1.0

Values are mean ± s.d. of six animals in each case.
Values in parentheses denote change (fold) with respect to control.

Table 3. Immunomodulatory activity of loganin analogues



Compd no. and structure	HA titre	Plaque forming cells	Macrophage migration index
13. R = OH, R^1 = H, R^2 = Ac, R^3 = CHO, R^4 = H	768 ± 391 (1.8)	161 ± 37 (1.4)	1.10 ± 0.28
14. R = OAc, R^1 = H, R^2 = Ac, R^3 = CH_2OH, R^4 = H	597 ± 39 (1.4)	115 ± 28 (1.0)	0.832 ± 0.12
15. R = OAc, R^1 = H, R^2 = H, R^3 = CH_2OH, R^4 = H	576 ± 322 (1.4)	103 ± 17 (0.9)	1.49 ± 0.18
16. R = OCOCH = CH–C$_6$H$_4$–OMe	682 ± 295	141 ± 33	0.86 ± 0.26
R^1 = H, R^2 = Ac, R^3 = CH_2OH, R^4 = OAc	(1.6)	(1.23)	
Control	426 ± 147	115 ± 25	1.0

Values are mean ± s.d. of six animals in each case.
Values in parentheses denote change (fold) with respect to control.

Lymphocyte transformation test (LTT). Splenocytes of normal mice were cultured in triplicate for three days in presence of 5, 10, 20 and 50 μg/mL concentration of 9 iridoid analogues in a flat bottom 96-well culture plate (Table 4). The culture was then pulsed with [^{3}H] thymidine (sp. activity 6600 mci/μM; BRIT, India), incubated further for 18 h and harvested. Radioactivity incorporated in the cells was measured using a β-liquid scintillation counter. The cells cultured without test compound were taken as control and the results have been expressed as transformation index (TI) which was calculated using formula.

$$\mathrm{TI} = \frac{\text{Mean CPM (experimental)}}{\text{Mean CPM (control)}}$$

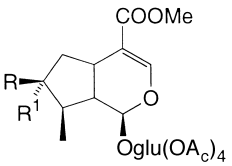
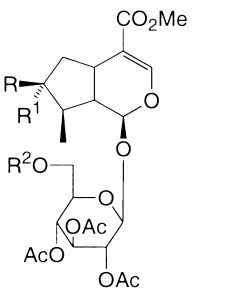
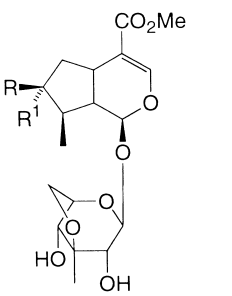
Optimization of the responses rendered by various doses are given in Table 4.

Results and Discussion

Immunostimulant activity (in vivo) associated with the starting materials namely loganin (**1**), ketologanin (**2**) and arbortristoside A (**3**) is described in Table 1. Compound **1** as compared to control enhanced HA titre by 3.3, MMI by 2.61 and PFC by 1.72-fold. Likewise compound **3** enhanced these parameters by 2.91, 2.61 and 3.95-fold, respectively. Ketologanin (**2**), to the contrary, did not exhibit immunostimulation. These results indicated that compounds **1** and **3** did not exhibit specificity for any component of the immune response and the oxidation of hydroxyl group at C-7 in the aglycon moiety almost completely abolished the immune stimulating property. The latter conclusion is further supported by the observation that ketologanin tetra acetate (compound **4**, Table 2) also did not show immune stimulating property. Unlike **4** loganin penta acetate (**5**, Table 2), however, exhibited elevated CMI and HI responses. MMI, HA titre and PFC counts for compound **5** were raised by 1.73-, 2.0- and 1.86-fold, respectively, as compared to control.

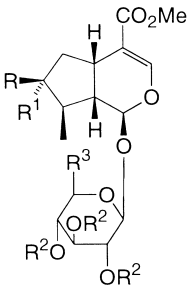
Single point modifications were carried out at C-6′ position in loganin (**1**) and 7-ketologanin (**2**) by elaborating these to various esters[16] viz. **6**–**9** (Table 2) and **19**–**20** (Table 4). First four compounds were tested only in vivo, but the latter two esters were tested in vivo and also in vitro (LTT) at different concentrations. Compared to the control, compound **8** enhanced the MMI by 1.6 fold and HA titre by twofold. Compounds **6** and **7** only moderately enhanced the MMI response but did not enhance PFC and HA titre (Table 2). The other two esters **19** and **20** although in vitro enhanced transformation index maximally to 2.0 at 10 μg/mL but failed to

Table 4. Inununomodulatory activity of loganin analogues

Compd no. and structure	HA titre	Plaque forming cells	Macrophage migration index	Lymphocyte proliferation (TI)*			
				5 μg	10 μg	20 μg	50 μg
							
17. R = H R^1 = OCOCH = CH—	682 ± 295 (426 ± 121,1.6)	125 ± 30 (115 ± 25,1.09)	1.23 ± 0.16	1.56	2.14	1.78	1.25
18. R = H R^1 = OCOCH = CH—	1536 ± 295 (1.6)	185 ± 66 (1.0)	1.74 ± 0.57	1.26	1.67	2.03	1.43
							
19. R,R^1 = O, R^2 = COCH = CH—	853 ± 170 (0.9)	136 ± 39 (0.7)	0.68 ± 0.31	1.76	2.16	1.31	1.13
20. R = OAc,R^1 = H R^2 = COCH = CH—	1365 ± 341	172 ± 25	1.80 ± 0.26	1.64	2.29	1.73	1.31
							
21. R = OH, R^1 = H	1365 ± 341 (1.4)	171 ± 38 (0.9)	1.0 ± 053	1.55	2.17	2.07	1.59
22. R,R^1 = O	682 ± 170 (0.7)	882 ± 39 (0.5)	0.79 ± 0.21	1.48	1.92	2.10	2.40

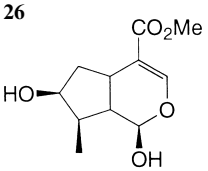
(*continued*)

Table 4—*contd*

Compd no. and structure	HA titre	Plaque forming cells	Macrophage migration index	Lymphocyte proliferation (TI)*			
				5 μg	10 μg	20 μg	50 μg
							
23. R = OAc, R^1 = H, R^2 = Ac, R^3 = CH_3,	1365 ± 341 (1.4)	163 ± 43 (0.85)	1.77 ± 0.88	1.24	1.30	1.37	1.28
24. R = OAc, R^1 = H, R^2 = Ac, R^3 = CH_2Cl,	597 ± 226 (0.6)	149 ± 61 (0.8)	1.35 ± 0.67	1.10	1.26	1.60	1.25
25. R = OH, R^1, R^2 = H, R^3 = CH_2NH_2	768 ± 256 (0.8)	144 ± 52 (0.8)	1.91 ± 0.83	1.25	1.57	1.14	1.10
Control	938 ± 85 (1.0)	181 ± 45	1	—	—	—	—

Values are mean ± s.d. of six animals in each case.
Values in parentheses denote change (fold) with respect to control.
*Values are mean of two experiments each done in triplicate.

Table 5. Immunomodulatory activity of loganin aglucon (**26**)

Compd no. and structure	HA titre	Plaque forming cells	Macrophage migration index	Lymphocyte proliferation (TI)*			
				5 μg	10 μg	20 μg	50 μg
26 	819 ± 114 (0.96)	109 ± 53 (0.908)	0.79 ± 0.40	1.3	0.82	0.66	0.84
Control	853 ± 108	120 ± 54	(1)	—	—	—	—

Values are mean ± s.d. of six animals in each case.
Values in parentheses denote change (fold) with respect to control.
*Values are mean of two experiments each done in triplicate.

show any impact on immune system in vivo (Table 4). Similarly single point modification at C-6′ position in ketologanin (**2**) carried out by introducing a trityl group (**10**, Table 2), revealed an interesting feature. The inactive ketologanin became immunostimulant as was evident from enhanced MMI, HA titre and PFC values. Acetylation of this compound yielded **11** which led to enhanced immunostimulant properties. Its HA titre and PFC counts were found to be more than loganin (**1**) but MMI value was slightly lesser. The involvement of C-6′ position in immune responses was observed again when detritylation of this compound (**11**) into **12** significantly reduced HA titre, the parameters of humoral response (Table 2).

The influence of ring configuration of glucopyranose structure on the immune response of the host was studied by monitoring the biological activities of compounds possessing a double bond between 4′,5′ position in β-D-glucopyranose unit (compounds **13**–**16**, Table 3). Another configurational perturbation brought about by preparing 3′,6′-anhydro sugar derivatives (compounds

21 and **22**, Table 4) gave the opportunity to study the influence of ring configuration for eliciting immune responses. The compounds of first group **13** to **16** were tested in vivo while of the latter (**21** and **22**) were tested both in vivo and in vitro. Out of the former four compounds 4′,5′-unsaturated aldehyde (**13**) selectively exhibited enhanced HA titre, but 4′,5′-unsaturated alcohols **14** and **16** led to suppressed MMI values. Amongst the latter two compounds tested in vitro **21** showed maximum transformation index at 10 μg/mL but **22** at 50 μg/mL (Table 4) and in vivo, **21** enhanced the HA titre by 1.4-fold and **22** depressed the HA titre by 0.7-fold. These two compounds did not show any change in PFC or MMI. When the functional group at C-6′ was replaced by methyl (**23**), chlorine (**24**) and amino (**25**), they did not show any remarkable change in their activity profile.

The modification at C-7 centre in the aglycon was concerned with stereochemistry, 7-epiesters (compounds **17** and **18**, Table 4) showed maximum transformation index of 2.14 at 10 μg/mL and 2.03 at 20 μg/mL, respectively. These compounds elevated HA titre and MMI values.

In order to clarify the role of sugar, the immunomodulatory activity of loganin aglycon (**26**, Table 5) was studied. It was observed that removal of the sugar moiety completely abolished the immunostimulant activity and led to mild immunosuppression as was evident from in vivo and in vitro studies.

The in vitro biological screening of the analogues of iridoid glycosides has revealed the need of an optimum concentration for the immunostimulant activity. As would be evident from Table 4, the immunostimulant activity after certain dose level decreases significantly. For example compounds **17**, **19**, **20**, **21**, and **25** showed highest transformation index (TI) at 10 μg/mL concentration but further increase in their concentration diminished the TI value, whereas **18**, **23** and **24** showed maximum transformation index (T1) at 20 μg/mL and compound **22** showed maxima at 50 μg/mL.

Conclusions

The locus at C-7 in the aglycon residue of iridoid glycosides plays a governing role for eliciting immunostimulant activity. In the sugar moiety, the locus at C-6 governs the immune response. Modifications at two loci namely unsaturation at 4′,5′-position and an aldehyde group at C-6′ position significantly enhanced the HA titre. In vitro results indicate that an optimum concentration in biophase may be essential for eliciting the immunostimulant activity.

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