Genetic Control of Serum Marinobufagenin in the Spontaneously Hypertensive Rat and the Relationship to Blood Pressure

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Background—We have investigated serum levels of immunoreactive marinobufagenin (MBG) in 16- to 20-week-old spontaneously hypertensive rats (SHRs)-A3 and in the normotensive Wistar-Kyoto (WKY) rat strain in the absence of salt loading, and we have investigated the genetic control of serum MBG.

Methods and Results—We genotyped the F2 progeny of an SHR-A3 × WKY intercross using a genome-wide panel of 253 single-nucleotide polymorphism markers that were dimorphic between SHR-A3 and WKY and measured serum MBG by ELISA. Serum MBG levels were lower in SHR-A3 than WKY rats (0.39 ± 0.07 and 1.27 ± 0.40 nmol/L, respectively), suggesting that MBG may not play a role in the markedly divergent blood pressure measured by telemetry in rats of these 2 strains (SHR-A3 and WKY, 198.3 ± 4.43 and 116.8 ± 1.51 mm Hg, respectively). The strain difference in serum MBG was investigated to determine whether genomic regions influencing MBG might be identified by genetic mapping. Quantitative trait locus mapping indicated a single locus influencing serum MBG in the region of chromosome 6q12. Homozygosity of WKY alleles at this locus was associated with increased serum MBG levels. We surveyed whole genome sequences from our SHR-A3 and WKY lines, seeking coding sequence variation between SHR-A3 and WKY within the mapped locus that might explain the inherited strain difference in serum MBG.

Conclusions—We identified amino acid substitution in the sterol transport protein Abcg5, present in SHR-A3, but absent in WKY, that is a potential mechanism influencing MBG levels. (J Am Heart Assoc. 2017;6:e006704. DOI: 10.1161/JAHA.117.006704.)

Key Words: cholesterol homeostasis • endocrinology • marinobufagenin • Na+/K+-ATPase • sitosterol

T here is ongoing debate about the chemical identity of an endogenous ligand for the evolutionarily conserved cardiotonic steroid (CS; “digitalis”) binding site that acts to inhibit the ion transport function of Na+, K+-ATPase and that may also induce cell signaling.1 An important area where increased knowledge is needed is identification of genes involved in the regulation and biosynthesis of this proposed endocrine material. At present, there is evidence that the CSs that can be detected in mammals by several indirect assays (functional and immunologic) may have an endogenous origin from steroid biosynthesis2–6 and may involve modification of the cholesterol side chain by CYP27A1.7 However, studies to investigate the genetic control of circulating CS have not been reported.

Genetic approaches may offer important opportunities to refine understanding of the origin and biosynthesis of CS. They may also explain interindividual variability in measured CS levels. Knowledge of biosynthetic and regulatory genes may also help clarify uncertainty about the chemical identity of CS present in mammalian serum. This field has been shaped by an early report that the mammalian CS was chemically identical to the structurally unusual (highly hydrophilic), plant-derived CS, ouabain.8 However, numerous independent investigations, including those using the most modern analytical chemistry methods, have shown that ouabain is not present in humans.9–11 We and others have developed evidence in support of similarity between CS and the vertebrate steroid, marinobufagenin (MBG).2,5–7,12–14 MBG is a CS aglycone synthesized by members of the Bufonidae family of true toads.15 Studies of cultured adrenocortical cells suggest MBG is also a product of mammalian adrenocortical steroidogenesis.2,3,5,6,13 However, definitive chemical and structural identification is incomplete. Use of cultured adrenocortical cells grown in defined media provides an opportunity to investigate endogenous origin. Also, it has provided evidence that material with MBG properties can be
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Clinical Perspective

What Is New?

• This article provides evidence that the endogenous production of the cardiotonic steroid marinobufagenin may be subject to inherited influences attributable to genetic variation in a sterol transport protein.

What Are the Clinical Implications?

• This work provides an initial genetic approach to uncover the mechanisms involved in regulation and biosynthesis of marinobufagenin.

detected and is related to cholesterol metabolism in adrenocortical cells. Specifically, we and others have shown that production of immunologically detected MBG is independent of cholesterol side chain cleavage, can be interrupted by blockade of de novo cholesterol synthesis, and appears to involve modification of the cholesterol side chain by a pathway known to be involved in bile acid synthesis.

In the present study, we report that immunologically detected MBG differs in abundance in the serum of inbred normotensive Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHRs). We have used genetic mapping in an SHR × WKY intercross to seek genomic loci that may be linked to such differences. The resulting mapping suggests that a narrowly defined locus on chromosome 6 influences serum MBG levels. We have examined genome sequence across SHR and WKY in this locus to identify genetic variation potentially related to serum MBG levels. We propose that the observations we have reported may be attributable to known functional genetic variation in sterol transport genes in this locus.

Methods

Animals

Studies were performed on male rats of the SHR-A3 (SHRSP/Bbb) and the normotensive WKY/Bbb inbred lines. These lines are maintained as closed colonies in our facility. The genetic integrity of the lines is verified using high-throughput genotyping of genome-wide single-nucleotide polymorphisms. Animals used in these studies were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International–approved animal facility with sentinel monitoring to confirm the absence of transmissible disease in the colony. They were provided a standard rodent chow diet and drinking water ad libitum. For mapping, SHR-A3 males were crossed with WKY females to generate the F1 progeny. F1 animals were brother-sister mated to create an F2 population from which 56 males (to avoid estrus cycle fluctuations in serum steroid hormones present in females) were selected for further study. Blood was collected into serum separator tubes by direct sampling from the abdominal aorta in anesthetized (3% isoflurane in oxygen by inhalation), laparotomized animals. Serum was collected after centrifugation and stored at −80°C.

Blood Pressure

Blood pressure (BP) was obtained from 16 SHR-A3 and 15 WKY animals. BP was measured by radiotelemetry in adult animals from 16 to 18 weeks of age. BP was also measured in the F2 progeny used for serum MBG estimation by the same method. Additional F2 animals were added, subsequently increasing the number of F2 animals from which BP measurements were available to 173. Catheters were implanted under isoflurane anesthesia into the abdominal aorta above the bifurcation and below the renal arteries. Animals were allowed to recover from implantation for at least 7 days before BP measurement began. After recovery, BP was measured for 24 hours. During each 24-hour recording, period pressures were sampled for 30 seconds every 30 minutes using DataQuest ART telemetry software.

Serum MBG Measurement

An ELISA was used for serum MBG measurement. Anti-MBG antibody production and purification was described previously in detail. Briefly, venom was collected from adult *Bufo marinus* toads, and MBG was purified by thin-layer chromatography and detected by chromatographic mobility and specific color reaction with antimony trichloride. The chemical structure of MBG was confirmed by mass spectrometry analysis. MBG-3-glycoside was synthesized, as described by Koenigs and Knorr, with some modifications. The scheme of MBG-protein preparation used in our study is given in Figure S1. MBG-3-glycoside bovine serum albumin conjugate was used for rabbit immunization, and MBG-3-glycoside-RNAase conjugate was used for coating of the solid phase in ELISA. Immunization protocols, MBG antibody purification procedures, and the results of anti-MBG antibody cross-reactivity analysis, as well as the linear range and detection limits, have been previously reported. For MBG measurements, serum samples from 10 SHR-A3, 9 WKY, and 56 F2 animals (aged 16–20 weeks) were prepared using C18 SepPak cartridges. Cartridges were activated with 10 mL of acetonitrile and washed with 10 mL water. Then, 1.0 mL of serum was applied to each cartridge, and the cartridge was rinsed with 7 mL of 25% acetonitrile, followed by extraction with 7 mL of 80% acetonitrile. The resulting extracts were dried under vacuum and stored at −80°C. Before
immunoassay, extracts were reconstituted in assay buffer and tested for their ability to inhibit the binding of rabbit anti-MBG antibody to solid-phase bound MBG (immobilized conjugate of MBG-3-glycoside to RNAse, 0.2 μg of conjugate in 0.1 mL of bicarbonate-buffered saline per well). We added 20 μL of MBG standards and unknown samples to the coated wells, followed by 80 μL of MBG antibody. After 1 hour of incubation, the wells were washed 3 times with 0.9% NaCl containing 0.05% Tween 20, after which 100 μL of secondary antibody was added (goat anti-rabbit IgG peroxidase). After 1 hour of incubation, the wells were washed 3 times and peroxidase substrate was added (TMB Microwell Peroxidase Substrate System). Optical density was read at 450 nm. The sensitivity of immunoassay was 0.0012 pmol per well. The cross-immunoreactivity of MBG antibody was as follows: MBG, 100%; digitoxin, 3.0%; bufalin, 1.0%; digoxin and cinobufagin, 1.0%; ouabain, 0.1%; and prednisone, spironolactone, proscillaridin A, progesterone, and pregnenolone, all <0.1%.

Genotyping

Single-nucleotide polymorphism genotyping was performed in DNA prepared from liver tissue in multiplex reactions using the Sequenom MassARRAY system. We selected ∼290 evenly spaced (average, ∼10Mbase between each marker) single-nucleotide polymorphism mapping markers known to be dimorphic in this cross (see Table S1 for information on marker locations throughout the genome). This resulted in successful automated single-nucleotide polymorphism genotyping calling from 253 of the selected markers. Mass spectrometry analysis of the extension reactions was performed using a Bruker Compact matrix-assisted laser desorption time-of-flight mass spectrometer. The mass spectra were collected, and SpectroCALLER software was used to automatically assign the genotype calls. Genotypes were obtained for sample parental line animals (SHR-A3 and WKY) and for all F2 animals.

Genetic Mapping

Mapping with R/qtl was performed using Haley-Knott regression to test the hypothesis that a single quantitative trait locus (QTL) could be identified that influences serum MBG levels.22,23 R/qtl was also used to estimate QTL effect sizes. The R/qtl version was 1.37-11.

Statistical Analysis

Serum MBG immunoreactive levels were obtained from 10 SHR-A3 and 9 WKY animals. Group results are described by mean±SEM. Group comparisons were performed using Student t tests (2 group) or using ANOVA, followed by post hoc pairwise comparisons using a Scheffé test (multigroup). Statistical analysis was performed using StatPlus:mac, Version 6.

Animal Welfare

All procedures involving the use of animals in this study were prospectively reviewed and approved by the Institutional Animal Care and Use Committee.

Results

Serum MBG levels in SHR-A3 and WKY rats were 0.39±0.07 and 1.27±0.40 nmol/L, respectively (Figure 1). Mean F2 serum MBG levels were 1.11±0.13 nmol/L. Systolic BP in SHR-A3 and WKY rats was 198.3±4.43 and 116.8±1.51 mm Hg, respectively (Figure 2). Average systolic BP in F2 animals was 144.1±1.01 mm Hg.

QTL mapping was performed to determine whether any genomic loci were linked to serum MBG levels. The resulting map is shown in Figure 3. QTL mapping provided suggestive evidence of a locus linked to serum MBG level that was centered on chromosome 6 (9.1 megabases), with a peak limit of detection score of 2.7 and the closest mapping marker being DSgcf11040 (Table S1). We investigated the effect of inheritance of SHR-A3 alleles at this chromosome 6 QTL on estimated serum MBG levels using R/qtl. Figure 4 shows that homozygoty or heterozygoty for SHR-A3 alleles (AA and AW in Figure 4) at this chromosome 6 QTL was associated with much lower levels of serum MBG than homozygoty for WKY alleles (WW in Figure 4). This suggests

![Figure 1](image-url)
a dominant effect of inheritance of chromosome 6 genetic variation arising from SHR at this locus to decrease serum MBG.

To determine whether genetic effects on serum MBG levels might be related to BP, we examined BPs in F2 animals in relation to the inheritance of SHR-A3 (A) or WKY (W) alleles at the chromosome 6 MBG QTL. In F2 animals whose genotypes at chromosome 6 (9.0 megabases) were SHR-A3 homozygous (AA), SHR-A3 heterozygous (WA), or WKY homozygous (WW), systolic BP levels were as follows: AA, 149.3±2.80 mm Hg; AW, 143.3±1.25 mm Hg; and WW, 140.4±1.77 mm Hg (ANOVA F=4.9107; P=0.0085; Fcrit=3.0533; Scheffé test P values for WW versus AA, P=0.011, WW versus WA, P=0.521, and WA versus AA, P=0.066.) Thus, BP effects may also be associated with genetic variation at this QTL. However, the direction of these effects does not provide a link between increased serum MBG and BP elevation in the SHR model.

We have used next-generation whole genome sequencing to identify rat genes lying in the QTL between markers at chromosome 6 (879 568 to 24 378 026) that contain amino acid substitutions in WKY or SHR-A3, compared with the rat reference genome sequence (Rn5). We found only 8 genes with coding sequence variation. These genes are identified in Table. The known functions of these genes have been considered for their potential role in modulating serum MBG levels and are considered in the Discussion.

**Discussion**

There is extensive evidence implicating CS in the pathogenesis of various models of salt-dependent hypertension and in the potential end-organ sequelae associated with such elevation of BP.24–29 We initiated these studies to determine whether CS might be relevant to BP levels in the widely used SHR model of hypertension. Although the SHR-A3 line does show additional elevation of BP after salt loading, severe hypertension is present in SHR in the absence of salt loading.30 Given the association between salt-sensitive BP elevation and CS levels, it may not be surprising that the SHR model does not reveal increased endogenous CS. However, it was unexpected to find significantly lower levels of CS in SHR-A3 compared with WKY rats. This suggested an opportunity to use genetic approaches to determine whether this difference
in serum MBG might be determined by genetic differences between SHR and WKY rats. An opportunity resulting from this line of investigation is to identify genetic variation affecting the biosynthesis or control of CS, with the potential to yield useful insight into important aspects of CS biochemistry and endocrinology.

We have previously developed evidence concerning the nature, cellular origin, and biosynthesis of mammalian material with properties similar to CSs. We have shown that the adrenocortical cell line Y-1, grown in a defined, serum-free medium, releases into the medium a material with chromatographic and mass spectrometric properties similar to the known vertebrate CS, MBG.2,3 MBG is an aglycone CS identified first as a defensive secretion of the parotid gland of members of the Bufonidae family, the common toads.15 Using Y-1 cell cultures, we have demonstrated that this material arises from the pathway in which cholesterol is biosynthesized. However, it differs from known steroid hormone products of the adrenal cortex in being produced by a pathway that does not involve cholesterol side chain cleavage.2 Recently, Fedorova and colleagues have shown that the CYP27A1-mediated modification of the cholesterol side chain, a modification that is in the pathway of bile acid formation, may be a step by which cholesterol is converted to MBG.7

SHR-A3 and WKY rats are inbred strains derived from outbred Wistar rats. It is well known that inbred lines derived from outbred Wistar rats may fix in homozygosity a variant of the Abcg5 gene. This variant has important functional consequences for the handling of cholesterol and dietary phytosterols and results in increased plasma total plant sterol levels.31 Our genome sequence analysis indicates that this Abcg5 variant is present in inbred SHR-A3 strain, but absent in our WKY strain (Table), which is derived from WKY/Izm.32 WKY/Izm is the only inbred WKY or SHR strain reported to lack this Abcg5 variation.33 Several facts point to the possibility that this Abcg5 variation is responsible for the difference in serum MBG observed between SHR-A3 and WKY rats. First, the Abcg5 variant maps directly under the chromosome 6 linkage peak we detected. Second, Abcg5 is the only gene in the locus in which variation has been previously linked to steroid hormone metabolism. Third, the only other gene in the locus previously linked to sterol metabolism that contains coding variation is Abcg8, which is known to function as a binding partner of Abcg5, with each protein contributing as a half transporter to the functional sterol transporter. We found that, in the F2 progeny, the effects of the SHR-A3 allele on serum MBG levels act in the direction expected from the MBG level in the parental strains.

Figure 4. Effect of inheriting spontaneously hypertensive rat (SHR)-A3 (A) and Wistar-Kyoto (WKY; W) alleles at chromosome 6 (9 megabases; marker name, DSgcf11040). Effect sizes were estimated in R/qtl using the “effectplot” function, which estimates weighted averages.23 Animals homozygous or heterozygous for SHR-A3 alleles (AA and AW) at DSgcf11040 had lower levels of serum marinobufagenin (MBG) than animals homozygous for WKY alleles (ANOVA F=5.15; Fcrit=3.17; P=0.009; Scheffé tests: WW vs AW, P=0.015; WW vs AA, P=0.025; and AA vs AW, P=0.956).

Table. Genes in the MBG QTL That Contain Amino Acid Substitutions

| Gene   | Chromosome 6 Gene Start Position | Coding Variant | Variant Strain* |
|--------|----------------------------------|----------------|-----------------|
| Cebpz  | 1 524 597                        | Leu418Ile      | WKY             |
|        |                                  | Ser962-Cys     | WKY             |
| Prkd3  | 1 604 557                        | Asn117Ser      | SHR-A3          |
|        |                                  | Ala511Thr      | SHR-A3          |
| Thumpd2| 3 762 198                        | Ile209Val      | SHR-A3          |
|        |                                  | Met542Val      | SHR-A3          |
| Haao   | 7 007 338                        | Ala267Val      | SHR-A3          |
| Thada  | 7 677 173                        | Gin427Lys      | WKY             |
|        |                                  | Asn1018Ser     | SHR-A3          |
|        |                                  | Ala1023Val     | WKY             |
| Abcg5  | 7 896 799                        | Gty583Cys      | SHR-A3          |
| Abcg8  | 7 897 005                        | Thr325Met      | WKY             |
|        |                                  | Leu344Ser      | WKY             |
| Lrpprc | 8 001 949                        | Asn249Ser      | SHR-A3          |
|        |                                  | Ser1020Ala     | WKY             |

MBG indicates serum marinobufagenin; QTL, quantitative trait locus; SHR, spontaneously hypertensive rat; and WKY, Wistar-Kyoto.

*Indicates which strain differs from the rat reference sequence.

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and result in lower serum MBG levels when present in F2 animals.

Functional studies of the effects of the SHR-A3 Abcg5 variant on sterol metabolism indicate several facts that may support a role for this variation in altered MBG levels in SHR-A3. First, the Abcg5 mutation present in SHR-A3 has been associated with reduced adrenal cholesterol content. The same phenotype of reduced adrenal cholesterol content has been observed in a more exaggerated degree with total loss of Abcg5 and Abcg8 function in a double-knockout mouse. Furthermore, humans with homozygous functional deletion of Abcg5 have adrenocortical insufficiency, with reduced cortisol responses to corticotropin stimulation. Furthermore, phytosterolemia results in inhibition of CYP27A1, an enzyme implicated in MBG biosynthesis, whereas salt loading appears to increase CYP27A1 adrenocortical abundance. These findings point to a possible mechanism of reduced MBG production by the adrenal glands in SHR-A3 compared with WKY animals in this study. The abnormal sterol handling induced by the Abcg5 mutation may reduce adrenal cholesterol availability and impede CYP27A1 activity; together, this may constrain biosynthesis of MBG in SHR-A3. Our genetic studies provide a rationale for the direction of future studies into MBG biosynthesis.

We have also found a relationship between inheritance of SHR-A3 alleles at the MBG QTL and systolic BP. The effect size on systolic BP of this locus is moderate, ≈13 mm Hg. This raises the question of whether this effect is also mediated by genetic variation in Abcg5. Because the standard rodent chow fed to our animals contains principally plant-derived nutrients and because the presence of the Abcg5 mutation in SHR-A3 is sufficient to produce phytosterolemia, it is possible that altered sterol metabolism in SHR-A3 compared with WKY may contribute to disturbed BP regulation. Existing evidence suggests that phytosterolemia may affect BP levels. In F2 animals from an SHR x Sprague Dawley cross in which Abcg5 wild-type and mutant alleles segregated, tail cuff measurements of BP indicated slightly, but not significantly, higher levels of BP in the F2 progeny inheriting homozygous mutant Abcg5 alleles compared with those inheriting homozygous wild-type Abcg5 alleles. The magnitude of this difference in 16-week-old animals was similar to that observed between our 16- to 20-week-old SHR-A3 and WKY animals. Effects on BP of dietary phytosterol intake in WKY rats harboring the Abcg5 mutation have also been investigated. Increasing intake by changing food phytosterol content from 0.2 to 2.1 g/kg lowered cholesterol content in tissues and increased BP by ≈12 mm Hg. Dietary phytosterol supplementation in stroke-prone SHR (SHR-A3) rats also resulted in increased BP and acceleration of stroke. The mechanism of BP changes of phytosterols in the presence of the Abcg5 mutation has not been elucidated, but may be related to effects of phytosterols on red blood cell deformability and platelet function. Phytosterolemia in Abcg5/Abcg8 knockout mice is associated with platelet abnormalities, including platelet activation and microparticle formation. Phytosterol loading in SHR and the resulting increase in BP are accompanied by altered renal gene expression of several genes implicated in BP.

In summary, we have identified a chromosome locus that influences serum MBG levels in SHR and that contains known functional variation in the important sterol transport protein encoded by Abcg5. The metabolic effects of this mutation may include effects to limit the availability of substrate and enzyme activity required for MBG biosynthesis; they also may also increase BP through other mechanisms. This work lends support to the importance of cholesterol as a precursor molecule in MBG synthesis in the adrenal glands and indicates Abcg5 as an important target for future investigation of the control of MBG production.

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Disclosures
None.

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Supplemental Material
Table S1. Markers and their positions used for SNP (single nucleotide polymorphisms) genotyping.

| SNP marker ID     | chromosome | position (Mbp) |
|-------------------|------------|----------------|
| gnlti56722        | 1          | 17.139         |
| g kob1330         | 1          | 26.531         |
| rdahlb1350        | 1          | 46.208         |
| gnlti93476        | 1          | 61.784         |
| gnlti01028        | 1          | 79.777         |
| gnlti52687        | 1          | 90.431         |
| gnlti08801        | 1          | 98.576         |
| rdahlb1356        | 1          | 101.758        |
| gnlti59517        | 1          | 125.888        |
| WKYs1203          | 1          | 132.962        |
| gnlti24582        | 1          | 139.403        |
| D Sga1621         | 1          | 157.604        |
| gnlti81974        | 1          | 162.245        |
| g kob177          | 1          | 185.342        |
| SHRSPr1927a       | 1          | 213.369        |
| rdahlb1630        | 1          | 220.638        |
| WKYs1241          | 1          | 234.502        |
| WKYs1454          | 1          | 249.482        |
| Cpn97077          | 1          | 252.197        |
| WKYGif1619        | 1          | 261.097        |
| Cpn30354          | 1          | 267.130        |
| SHRSPr1675        | 2          | 2.892          |
| SHRSPr174         | 2          | 11.549         |
| Cpn75992          | 2          | 20.176         |
| SHRSPr11037       | 2          | 28.781         |
| WKYs1422          | 2          | 48.056         |
| gnlti91117        | 2          | 65.243         |
| WKYOas1345        | 2          | 79.640         |
| DahlSbr169        | 2          | 102.154        |
| WKYr1498          | 2          | 120.104        |
| SHRSPr1316        | 2          | 121.243        |
| WKYOas1515        | 2          | 139.958        |
| gnlti28177        | 2          | 141.186        |
| WKYOas160         | 2          | 168.205        |
| g kob1578         | 2          | 173.970        |
| rdahlb1103        | 2          | 183.996        |
| g kob194          | 2          | 192.803        |
| ratcb629          | 2          | 198.234        |
| g kob1212a        | 2          | 213.692        |
| SHRSPr1538        | 2          | 219.012        |
| g kob1518         | 2          | 225.693        |
| ratcb486          | 2          | 242.876        |
| ratca608          | 2          | 247.061        |
| g kob1189         | 2          | 256.708        |
| gnlti89021        | 3          | 6.445          |
| ratcb145          | 3          | 13.643         |
| Term               | Value | Score |
|--------------------|-------|-------|
| gkob1513           | 3     | 21.453|
| WKYr1208           | 3     | 31.460|
| gkob1490           | 3     | 40.454|
| WKYGif1184         | 3     | 50.028|
| gnlti73066         | 3     | 57.503|
| ratca483           | 3     | 67.663|
| Cpn06547           | 3     | 76.607|
| SHRSPr1273         | 3     | 95.587|
| gkob1374           | 3     | 101.235|
| Cpn34396           | 3     | 118.129|
| Cpn92684           | 3     | 122.593|
| WKYs1575           | 3     | 126.238|
| gkob1320           | 3     | 139.446|
| WKYs1653           | 3     | 148.888|
| gnlti56655         | 3     | 156.297|
| gnlti81100         | 3     | 167.427|
| WKYGif1253         | 4     | 3.713 |
| Cpn74882           | 4     | 9.075 |
| SHRSPr1685         | 4     | 21.334|
| gkob1792           | 4     | 28.965|
| gkob165a           | 4     | 40.690|
| gkob1638           | 4     | 46.591|
| WKYs1261a          | 4     | 55.671|
| WKYs1381           | 4     | 65.965|
| rdahlb1302         | 4     | 73.796|
| ratca224           | 4     | 83.360|
| ratca663           | 4     | 106.595|
| DSgc1275           | 4     | 119.414|
| gnlti49591         | 4     | 140.301|
| rdahlb1462         | 4     | 141.041|
| gnlti53423         | 4     | 147.616|
| gkob1292           | 4     | 157.496|
| WKYs1630           | 4     | 162.204|
| ratca208a          | 4     | 173.911|
| WKYGif1829         | 5     | 0.144 |
| gkob1295           | 5     | 8.433 |
| Cpn16274           | 5     | 17.516|
| SHRSPs1302         | 5     | 26.908|
| gnlti41173         | 5     | 31.072|
| gkob1521           | 5     | 48.841|
| gkob1342           | 5     | 54.801|
| WKYs1417           | 5     | 61.101|
| gkob1181a          | 5     | 68.069|
| SHRSPs1440         | 5     | 99.335|
| gkob1311           | 5     | 106.408|
| Cpn39002           | 5     | 108.839|
| gkob1337a          | 5     | 141.745|
| Gene     | Chromosome | Position |
|----------|------------|----------|
| WKYs1615 | 5          | 145.032  |
| SHRSPr1271 | 5      | 148.385  |
| gnlti50648 | 5       | 150.352  |
| Cpn01903  | 5          | 153.702  |
| rdahlb1378 | 5       | 162.338  |
| WKYr176   | 6          | 1.068    |
| DSgcf11040 | 6       | 9.010    |
| WKYs1663  | 6          | 22.443   |
| Cpn73879  | 6          | 27.474   |
| gkob1201  | 6          | 37.442   |
| WKYr1526  | 6          | 45.740   |
| gnlti43349 | 6       | 55.339   |
| DSgaf1128 | 6          | 64.251   |
| gnlti80969 | 6       | 75.043   |
| WKYGfr1414 | 6      | 82.482   |
| ratca431  | 6          | 94.397   |
| WKYGj1232 | 6          | 100.597  |
| gnlti87595 | 6       | 106.354  |
| DSgcf1155 | 6          | 118.530  |
| Cpn70553  | 6          | 126.671  |
| WKYs1104  | 6          | 136.628  |
| SHRSPs1372 | 6      | 145.120  |
| rdahlb1145 | 7       | 1.535    |
| WKYs1443  | 7          | 11.598   |
| WKYOas1463a | 7    | 17.684   |
| rdahlb1616 | 7       | 28.662   |
| rdahlb1496 | 7       | 45.056   |
| gkob1551  | 7          | 59.291   |
| gkob1237  | 7          | 64.708   |
| gkob1167  | 7          | 70.298   |
| gkob1281  | 7          | 95.133   |
| Cpn11739  | 7          | 101.412  |
| Cpn65618  | 7          | 113.866  |
| ratca161  | 7          | 117.117  |
| gnlti07347 | 7       | 128.153  |
| ratca534  | 7          | 135.139  |
| gkob1224  | 8          | 0.966    |
| gkob1488  | 8          | 9.921    |
| gkob2120  | 8          | 18.990   |
| rdahlb1212 | 8       | 48.410   |
| SHRSPr1758 | 8      | 69.618   |
| DSgc1111  | 8          | 73.480   |
| gkob1164  | 8          | 80.822   |
| gnlti54929 | 8       | 91.557   |
| Cpn72345  | 8          | 99.572   |
| WKYs1487  | 8          | 120.626  |
| ratca96   | 8          | 124.882  |
|     |     |       |
|-----|-----|-------|
| Cpn52115  | 8   | 127.952 |
| gnlit85693 | 9   | 24.414  |
| SHRSPPr1558 | 9   | 37.887  |
| SHRSPPr1297 | 9   | 42.237  |
| SHRSPPr1598 | 9   | 52.763  |
| gkob124     | 9   | 61.392  |
| SHRSPPr1375 | 9   | 87.238  |
| WKYOar1123  | 9   | 100.798 |
| WKYs1321a   | 9   | 108.212 |
| SHRSPPr1102 | 10  | 9.202   |
| rdahlb1251  | 10  | 18.102  |
| WKYs1755    | 10  | 28.465  |
| ratca65     | 10  | 31.783  |
| SHRSPs194   | 10  | 50.587  |
| ratca422    | 10  | 54.401  |
| DSgaf1646   | 10  | 61.877  |
| rdahlb1507  | 10  | 71.032  |
| Cpn04941    | 10  | 88.105  |
| gkob1512    | 10  | 99.462  |
| gkob1464    | 10  | 108.586 |
| ratca310a   | 11  | 0.258   |
| DSgaf1726   | 11  | 9.563   |
| DSgar155    | 11  | 18.295  |
| rdahlb1155  | 11  | 28.129  |
| ratca480    | 11  | 36.467  |
| gkob1277    | 11  | 41.325  |
| gkob172     | 11  | 54.440  |
| rdahlb126   | 11  | 63.554  |
| SHRSPPr1554 | 11  | 80.282  |
| SHRSPs1371  | 12  | 2.418   |
| WKYs1582    | 12  | 11.382  |
| ratca591    | 12  | 20.845  |
| SHRSPPr127  | 12  | 29.640  |
| gnlit69184  | 12  | 39.299  |
| rdahlb11110 | 13  | 8.519   |
| WKYr1204    | 13  | 17.103  |
| Cpn15422    | 13  | 30.915  |
| gnlit81740  | 13  | 35.603  |
| ratca430    | 13  | 53.175  |
| WKYOar1256a | 13  | 62.233  |
| Cpn21290    | 13  | 81.321  |
| gkob1102    | 13  | 95.095  |
| WKYr1376    | 13  | 110.912 |
| Cpn26240    | 14  | 2.826   |
| WKYOas1110  | 14  | 18.900  |
| ratca388    | 14  | 21.022  |
| gnlit23218  | 14  | 27.803  |
| Sample ID       | Chromosome | Value  |
|-----------------|------------|--------|
| rdahlb1187      | 14         | 44.785 |
| ratca218a       | 14         | 45.560 |
| Cpn55096        | 14         | 70.755 |
| WKYr1625        | 14         | 84.593 |
| WKYs1248        | 14         | 88.707 |
| gnlti27885      | 14         | 103.145|
| SHRSPr1301      | 15         | 11.885 |
| SHRSPs1102      | 15         | 22.224 |
| gnlti88742      | 15         | 26.987 |
| gkob174         | 15         | 39.063 |
| gkob1144        | 15         | 46.730 |
| SHRSPs1572      | 15         | 57.814 |
| rdahlb1664      | 15         | 75.215 |
| gkob1535        | 15         | 84.264 |
| gkob1396        | 15         | 93.740 |
| gkob1254        | 15         | 99.647 |
| Cpn52335        | 15         | 106.252|
| WKYs1198        | 16         | 0.569  |
| WKYr174         | 16         | 9.131  |
| ratca254a       | 16         | 18.825 |
| gkob1215        | 16         | 28.247 |
| gnlti70071      | 16         | 36.796 |
| rdahlb1205      | 16         | 45.679 |
| WKYs1300a       | 16         | 54.121 |
| Cpn88473        | 16         | 64.888 |
| WKYGjr163       | 16         | 71.648 |
| WKYr1437        | 16         | 81.655 |
| gkob1213        | 16         | 89.728 |
| WKYOas1201      | 17         | 2.911  |
| Cpn54082        | 17         | 12.454 |
| DSgaf1152       | 17         | 19.559 |
| Cpn15864        | 17         | 28.816 |
| WKYGjr1185      | 17         | 48.097 |
| gnlti14648      | 17         | 58.841 |
| gkob1218        | 17         | 75.204 |
| gkob167         | 1          | 35.248 |
| gkob1343        | 17         | 92.009 |
| DSgcfl1162      | 18         | 3.122  |
| J1308870        | 18         | 6.216  |
| WKYs1353a       | 18         | 18.061 |
| gkob1194        | 18         | 33.755 |
| SHRSPr1458      | 18         | 54.454 |
| SHRSPr1494      | 18         | 72.472 |
| gkob1612        | 19         | 5.493  |
| gkob1120        | 19         | 7.533  |
| WKYs1364        | 19         | 18.638 |
| SHRSPr1649      | 19         | 27.966 |
| gkob1192  | 19 | 37.850 |
g| gkob1133  | 19 | 45.850 |
ratca460  | 20 | 0.035  |
ratca559  | 20 | 8.901  |
WKYs1124  | 20 | 19.701 |
SHRSPs156 | 20 | 27.269 |
ratca621  | 20 | 38.643 |
WKYr1292  | 20 | 45.513 |
SHRSPr1498| 20 | 54.228 |
SHRSPs189 | X  | 3.967  |
WKYGj11223| X  | 14.504 |
Cpn54944 | X  | 21.255 |
WKYr1841 | X  | 37.124 |
gntii7424| X  | 55.167 |
rdaahlb1173| X  | 103.023 |
J500540a | X  | 112.484 |
WKYGgr177| X  | 134.806 |
gkob1389  | X  | 138.594 |
**Figure S1.** Pathway of synthesis of marinobufagenin assay reagents. **A)** Synthesis of marinobufagenin-3-glycoside. **B)** Mass spectrometry analysis of marinobufagenin-3-glucoside. **C)** Wohl degradation of glycoside ring to allow coupling to protein. **D)** Borohydride reductive coupling to bovine serum albumin.