C_{10}-O_{eq}-N-(4-AZIDO-5-^{125}IODO SALICYLOYL)-β-ALANYL--β ALANYL RYANODINE (Az-βAR), A NOVEL PHOTO-AFFINITY LIGAND FOR THE RYANODINE BINDING SITE

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SUMMARY

A high affinity, photoactivatable, radio-iodinated ligand for the ryanodine binding site(s) of the sarcoplasmic reticulum calcium-release channel, C_{10}-O_e-N-(4-azido-5-^{125}iodo salicyloyl)-β-alanyl-β-alanyl ryanodine (Az-βAR), was synthesized at a specific activity of 1400mCi/mmol. Prepared by condensing the versatile synthon, N-(4-azido-5^{125}iodo salicyloyl)-β-alanine with C_{10}-O_{eq}-β-alanyl ryanodine, Az-βAR, like [^{3}H] ryanodine, does not demonstrate saturation binding kinetics. ^{127}Az-βAR exhibits an IC_{50} of 27.2 ± 2 x10^{-9} M (mean ± SD) compared to ryanodine's 6.2 ± 0.4 x10^{-9} M for the ryanodine receptor/calcium release channel of sarcoplasmic reticulum vesicles isolated from rabbit skeletal muscle.

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INTRODUCTION

The neutral alkaloids ryanodine and 9,21-didehydro-ryanodine, isolated from the tropical shrub *Ryania speciosa* Vahl are specific and potent effectors of the sarcoplasmic reticulum calcium-release channel (SR-CRC/ryanodine receptor) of striated muscle (1). In calcium flux experiments using rabbit skeletal heavy SR vesicles, these congeners display two distinct and opposite actions. At concentrations ranging from 10nM to 30μM they activate (open) the CRC, whereas concentrations greater than that required for maximal activation, deactivate (functionally close) the CRC. These unique characteristics have made the ryanoids useful as tools to investigate the role of intracellular Ca^{2+} in the excitation-contraction coupling process of muscle cells (2-7) .

Although significant progress has been made in characterizing the Ca^{2+} regulated contractile machinery of muscle cells (8-10), little is certain about the molecular basis of the interactions that prompt modulation of the SR-CRC by these compounds. We report here the first synthesis of a high affinity, photoactivatable, radio-iodinated derivative of ryanodine, C_{10}-O_{eq}-N-(4-azido-5-^{125}iodo salicyloyl)-β-alanyl-β-alanyl ryanodine (Az-βAR) that should be useful in elucidating the ryanoids' actions on the SR-CRC. In addition, this novel derivative should aid in localizing or pinpointing the ryanodine binding site(s) in other tissues such as the Purkinje cells of the brain, chromaffin cells of the adrenal cortex, the liver and neutrophils (11-15).

In the present study, 4-amino salicylic acid and 4-azido salicylic acid N-hydroxy succinimide ester each were converted to the synthon N-(4-azido-5-^{125}iodo salicyloyl)-β-alanine. Condensation of this synthon with C_{10}-O_{eq}-β-alanyl ryanodine under mild conditions afforded Az-βAR.

MATERIALS

[^{3}H] ryanodine was purchased from Du Pont-New England Nuclear (Boston, MA). Na^{125}I was obtained from Amersham (Arlington Heights, IL). Wood from *Ryania speciosa* Vahl was supplied by Integrated Biotechnology Corporation (Carmel, IN). Ryanodine was isolated in our laboratory from the ground stems of *Ryania* wood. In brief, presoaked wood (800g powder/200mL saturated sodium bicarbonate) was exhaustively extracted (soxhlet) for 24-36 hr with ethyl acetate (EtOAc). The extract was filtered, rotary evaporated to dryness, and redissolved in methylene chloride (CH_2Cl_2)/EtOAc (50:50). Three such extracts were pooled and chromatographed on silica gel (425g, Sigma 70-230 mesh), washing with 1200mL each of CH_2Cl_2, CH_2Cl_2/EtOAc (3:1), and CH_2Cl_2/EtOAc (1:1) to remove interferents. The ryanoids remaining on the column were then eluted with 1500mL EtOAc. The eluate was rotary evaporated to dryness and taken up in 8mL methanol. The individual ryanoids were separated using semi-preparative high performance liquid chromatography (mobile phase: methanol/ water, 43:57) and freeze dried from dioxane (Figure 1). Final purity of the ryanoids were ≥ 99%. All other chemicals used were of the highest purity available commercially.

CHEMISTRY

Two methods were used to prepare Az-βAR (Schemes 1 and 2), via 4-amino salicylic acid or 4-azido salicylic acid N-hydroxy succinimide ester to prepare the synthon, 4-azido-5-^{125}iodo salicyloyl-β-alanine. Subsequent condensation of the latter with C_{10}-O_{eq}-β-alanyl ryanodine afforded Az-βAR. These reactions (Schemes 1 and 2) were initially carried out using non-radioactive iodine and the reaction intermediates were characterized by ^{1}H NMR (300 MHz), mass spectrometry (FAB) and infrared spectroscopy. During the radio-synthesis, each non-radioactive intermediate was used as an internal standard to detect/confirm the

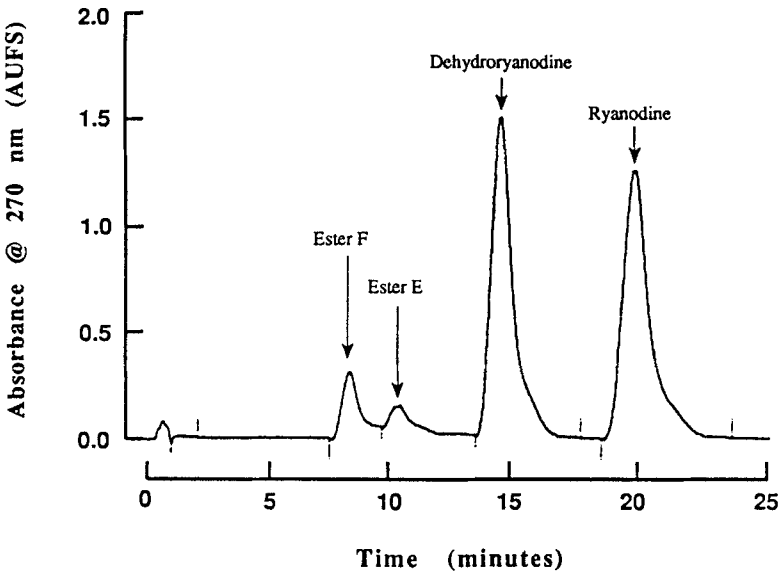


Fig. 1 HPLC chromatogram of the ethyl acetate extract of *Ryania speciosa* wood, after preliminary purification by column chromatography on silica gel. The minor secondary metabolites *Ryania* diterpene ester E and ester F, have previously been described (31).

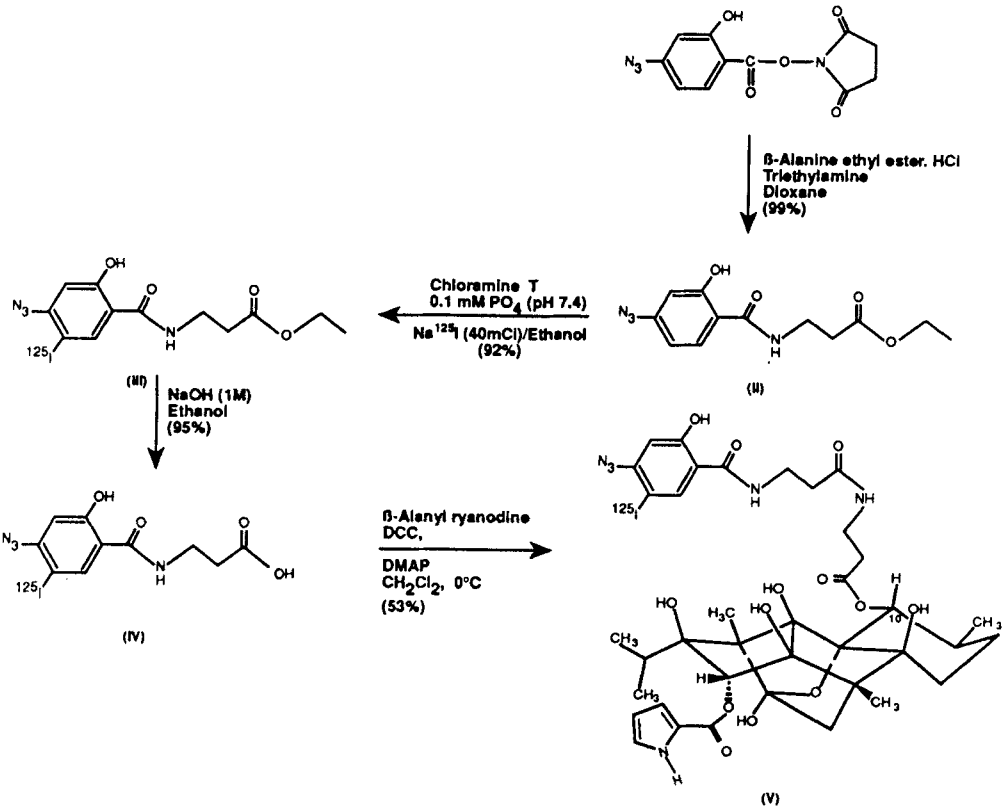
product of interest in the schemes, using thin layer chromatography. All reactions with azido reagents were carried out in subdued light to minimize photodegradation. C_{10}-O_{eq}-β-alanyl ryanodine was prepared as described previously (16).

EXPERIMENTAL

(A) Preparation of Az-βAR, starting from 4-azido salicylic acid N-hydroxysuccinimide ester. Four steps were required to obtain the desired product (Scheme 1).

N-(4-azido salicyloyl)-β-alanine ethyl ester (II). To a stirred solution of 4-azido salicylic acid N-hydroxy succinimide ester (100mg, 0.36 mmol in dioxane), β-alanine ethyl ester.HCl (62mg, 0.41 mmol) and triethylamine

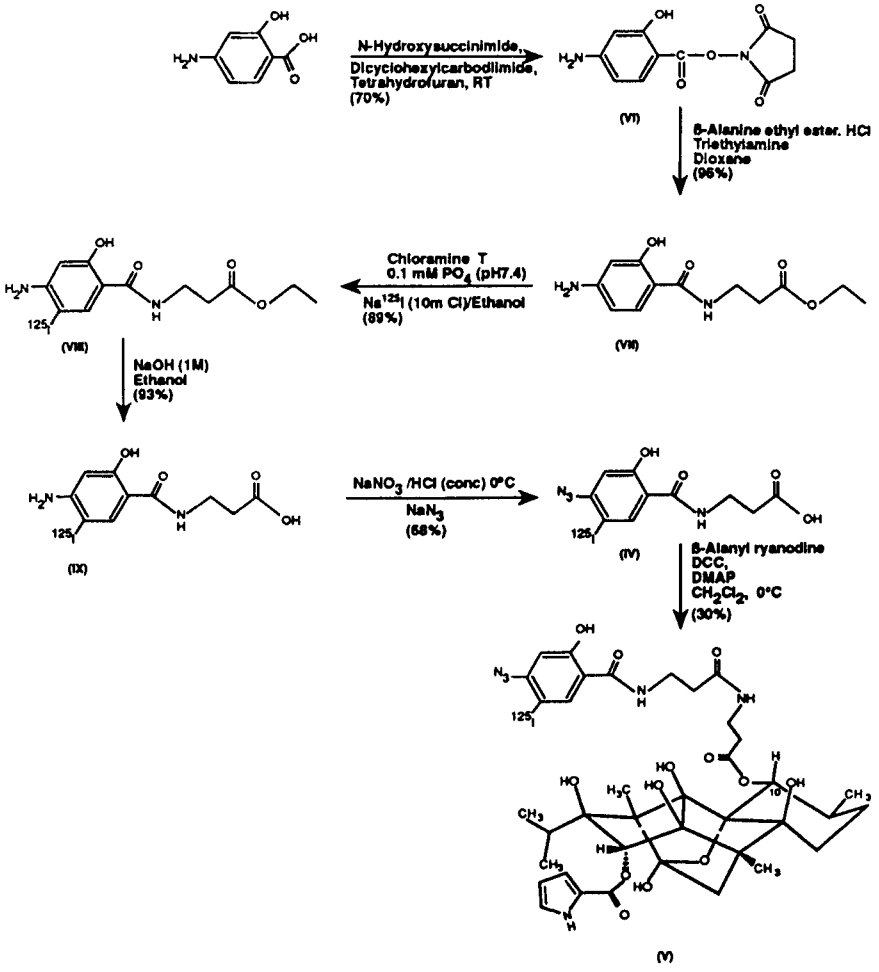
Scheme 1: Synthesis of C_{10}-O_{eq}-N-(4-azido-5-^{125}iodo salicyloyl β-alanyl-β-alanyl ryanodine starting from 4-azido salicylic acid N-hydroxysuccinimide ester.



(TEA, 0.5mL, 0.4 mmol) were added. After 1 hr, the reaction solvent was removed, the residue redissolved in chloroform ($CHCl_3$, 2mL) and chromatographed on silica gel (18g, mesh100-200, Type 60Å, Mallinkcrodt). The product, II, eluted with $CHCl_3$/1% methanol (MeOH). A total of 100mg (99% yield) of II was obtained, TLC R_f=0.73 ($CHCl_3$/MeOH, 85:15). IR(nujol) 2102cm^{-1} (azide)

N-(4-azido-5-^{125}iodo salicyloyl)-β-alanine ethyl ester (III). Iodination was performed using the procedure of Mais et al. (17). To II (10.0mg, 0.04 mmol in absolute ethanol, EtOH), sodium iodide (NaI, 6.75mg, 0.045 mmol) and 300μL Na^{125}I (30mCi) were added and the solution

Scheme 2: Synthesis of C_{10}-O_{eq}-N-(4-azido-5-^{125}iodo salicyloyl β-alanyl-β-alanyl ryanodine starting from 4-amino salicylic acid



allowed to stir for 2 min. Chloramine T (12.0mg, 0.52 mmol in 0.1M PO_4 buffer, pH 7.4, freshly prepared) was added as a bolus and the mixture stirred vigorously for 30 sec. The reaction was terminated by the addition of 0.5mL of 5% sodium metabisulphite (freshly prepared). The solution was extracted with 6x15mL $CHCl_3$ and the pooled extracts were dried over anhydrous sodium sulphate (Na_2SO_4), filtered and rotary evaporated to dryness. The residue was dissolved in $CHCl_3$ and

chromatographed on silica gel (10g). The product, III eluted with $CHCl_3$/2% MeOH. On freeze drying from dioxane 13.4mg (92% yield) of III was obtained, TLC R_f=0.56 ($CHCl_3$/MeOH, 85:15). (Non-radioactive compound, i.e. N-(4-azido-5-^{127}iodo salicyloyl)-β-alanine ethyl ester: IR (nujol) 2095cm^{-1} (azide), ^{1}H NMR ($CDCl_3$) δ 7.74 (s, aromatics), δ 6.65 (s, aromatics), δ 4.24 (q, 2H), δ 3.74 (m, 2H), δ 2.64 (m, 2H), δ 1.3 (t, 3H). The two sharp singlets at δ 7.74 and δ 6.65 establish that the iodine atom has been incorporated para to the hydroxyl moiety on the azidosalicyl group.

N-(4-azido-5-^{125}iodo salicyloyl)-β-alanine (IV). Sodium hydroxide (1mL, 1.0M) was added to III (13.4mg, 0.033 mmol, in EtOH) and the solution stirred for 8 hr. The mixture was acidified with hydrochloric acid (HCl, 2M, pH 1.0) and extracted with 6x15mL $CHCl_3$. The pooled extracts were dried (Na_2SO_4), filtered, rotary evaporated to dryness, redissolved in dioxane and freeze dried. Compound IV (11.8mg, 95% yield) was obtained, TLC R_f=0.0 ($CHCl_3$/MeOH 85:15) and R_f=0.72 ($CHCl_3$/MeOH/formic acid (HCOOH), 85:14.5:0.5). (Non-radioactive IV, IR (nujol) 2100cm^{-1} (azide), MS (FAB) *m/z* 376.9, $(M+H)^+$, calcd mol. wt. ($C_{10}N_4O_4H_9I$)=376.1, ^{1}H NMR ($CDCl_3$) δ 7.81 (s, aromatics), δ 6.70 (s, aromatics), δ 3.62 (m, 2H), δ 2.58 (m, 2H)).

C_{10}-O_{eq}-N-(4-azido-5-^{125}iodo salicyloyl)-β-alanyl-β-alanyl ryanodine (Az-βAR, V). C_{10}-O_{eq}-β-alanyl ryanodine was prepared as described previously (16). Briefly, ryanodine was coupled at the C_{10} hydroxyl to N-carbobenzoyl -oxy-β-alanine using dicyclohexylcarbodiimide (DCC) and dimethylamino-pyridine (DMAP) in CH_2Cl_2 at 0^{0}C for 7 hr. Hydrogenolysis of the product, C_{10}-O_{eq}-carbobenzoyloxy-β-alanyl ryanodine in ethanol using 10% Pd-on-C and triethylamine afforded C_{10}-O_{eq}-β-alanyl ryanodine.

To a solution of IV, (11.8mg, 0.032 mmol in CH_2Cl_2), C_{10}-O_{eq}-β-alanyl ryanodine (20.0mg, 0.035 mmol), DMAP (4mg, 0.04 mmol) and DCC (20mg, 0.05 mmol) were added and the reagents, maintained under

continuous stirring, were allowed to react for 7 hr at 0^0C. The reaction was stopped by adding 1mL of water to the solution and the solvents were rotary evaporated. The residue was redissolved in $CHCl_3$ and chromatographed on silica gel (10g). The product of interest, Az-βAR eluted with $CHCl_3$/6% MeOH. On rotary evaporation and freeze drying from dioxane, 17.5mg (53% yield), TLC R_f=0.30 ($CHCl_3$/MeOH/aqueous methylamine, ($MeNH_2$), 85:14:1) was obtained. ^{127}IAz-βAR, IR (nujol) 2105cm^{-1} (azide), *m/z* $(M+Li)^+$ 929.7, calcd. mol. wt. ($C_{38}N_6O_{13}H_{47}I$) =922.7. The specific activity of Az-βAR was 1400mCi/mmol, (a total of 26.1mCi was incorporated into the product) .

(B) Preparation of Az-βAR starting from 4-amino salicylic acid. Beginning with this reagent, six reaction steps were required to obtain the desired product, not including the synthesis of C_{10}-O_{eq}-β-alanyl ryanodine (Scheme 2).

4-amino salicylic acid N-hydroxysuccinimide ester (VI). To a stirred solution of 4-amino salicylic acid (2.07g,13 mmol in anhydrous tetrahydrofuran), N-hydroxysuccinimide (2.07g, 18 mmol) and DCC (3.44g,18 mmol) were added sequentially and allowed to react at 0^0C. After 7 hr the solvent was removed, the residue redissolved in $CHCl_3$/acetonitrile (CH_3CN) (98:2) and chromatographed on silica gel (80g). Compound VI eluted in $CHCl_3$/10% CH_3CN. A total of 2.4g (70% yield) of purified VI was obtained, TLC R_f=0.64 ($CHCl_3$/CH_3CN, 87:13).

N-(4-amino salicyloyl)-β-alanine ethyl ester (VII). To a stirred solution of VI (1.0g, 4 mmol in dioxane) and β-alanine ethyl ester.HCl (0.76g, 5 mmol), triethylamine (5.0mL, 4.0 mmol) were added. After 2hr the solvent was rotary evaporated and the residue was redissolved in $CHCl_3$ and chromatographed using silica gel (50g). Compound VII

eluted with $CHCl_3$/2% MeOH. A 96% yield was obtained, TLC R_{fs}=0.54 and 0.50 in $CHCl_3$/MeOH/$MeNH_2$ (85:14.5:0.5) and $CHCl_3$/acetone $(CH_3)_2CO$) (80:20) respectively. ^1H NMR ($CDCl_3$) δ 7.55 (d, aromatics), δ 6.35 (s, aromatics), δ 6.41(s, aromatics), δ 4.24 (q, 2H), δ 3.44 (q, 2H), δ 2.55 (q, 2H), δ 1.3 (t, 3H).

N-(4-amino-5-^{125}iodo salicyloyl)-β-alanine ethyl ester (VIII). To a stirred solution of VII(10.0mg, 0.033 mmol in EtOH), NaI (5.2mg, 0.035 mmol) was added, followed by 100μL $Na^{125}I$ (10mCi). Chloramine T (8.0mg, 0.035 mmol in 0.1M PO_4 buffer, pH 7.4, freshly prepared) was added as a bolus. After 30 sec the reaction was stopped by the addition of 5% sodium metabisulphite (0.5mL). The mixture was extracted with 5x15mL $CHCl_3$, and the pooled $CHCl_3$ extracts were dried over Na_2SO_4, filtered and rotary evaporated. The residue was redissolved in $CHCl_3$ and chromatographed on silica gel (10g). Compound VIII eluted with $CHCl_3$/1.5% MeOH. Upon rotary evaporation and freeze-drying from dioxane, 13.2mg (89.3% yield) of VIII was obtained, TLC R_f=0.62 in $CHCl_3$/MeOH/$MeNH_2$ (85:14.5:0.5).

N-(4-amino-5-^{125}iodo salicyloyl)-β-alanine (IX). To VIII (13.2mg, 0.031 mmol in EtOH) NaOH was added and the suspension stirred for 10hr. HCl (10 mL, 2M, pH1) was added and the solution extracted with 5x15mL $CHCl_3$. The pooled $CHCl_3$ layers were dried (Na_2SO_4), filtered and rotary evaporated. On freeze-drying from dioxane, 11.5mg (93% yield) of N-(4-amino-5-^{125}iodo salicyloyl)-β-alanine, IX was obtained, TLC R_f=0.52 in $CHCl_3$/MeOH/HCOOH (85:14.5:0.5). (Non-radioactive IX, ^1H NMR ($CDCl_3$), δ 7.75 (d, aromatics), δ 6.65 (s, aromatics), δ 6.65 (s, aromatics), δ 3.74 (m, 2H), δ 2.64 (m, 2H).

N-(4-azido-5-^{125}iodo salicyloyl)-β-alanine (IV). The synthesis of N-(4-azido-5-^{125}iodo salicyloyl)-β-alanine was conducted using the procedure described by Kiehem and Ji (18). To a suspension of VIII (11.5mg, 0.029

mmol in conc. HCl in ice bath), sodium nitrite (6.0mg, 0.087 mmol in cold distilled water) was added and the mixture stirred for 30 min. Sodium azide (10mg, 0.15 mmol in distilled water) was added very slowly to this suspension, maintaining continuous stirring and a reaction temperature of 0^0C. After 3 hr, the solution was extracted with 4x15mL $CHCl_3$. The pooled $CHCl_3$ extracts were dried over Na_2SO_4, filtered and rotary evaporated. The residue was redissolved in $CHCl_3$ and chromatographed using silica gel (10g). Compound IV eluted with $CHCl_3$/4% MeOH. Upon rotary evaporation, redissolving in $CHCl_3$, drying over Na_2SO_4, filtering, evaporating to dryness, and freeze-drying from dioxane, 8.1mg (67.8% yield) of IV, TLC R_f=0.72 $CHCl_3$/MeOH/ HCOOH (85:14.5:0.5) was obtained. (Non-radioactive IV, IR 2100cm^{-1} (azide), *m/z* $(M+H)^+$ 376.9, calcd. mol. wt. ($C_{10}N_4O_4H_9I$)=376.1, ^{1}H NMR δ 7.80 (s, aromatic), δ 6.70 (s, aromatic), δ 3.60 (q, 2H)), δ 2.55 (q, 2H)).

C_{10}-O_{eq}-N-(4-azido-5-^{125}iodo salicyloyl)-β- alanyl-β-alanyl ryanodine (Az-βAR, V). Compound IV (8.1mg, 0.02 mmol) in dried CH_2Cl_2 was added dropwise to a continuously stirred solution of C_{10}-O_{eq}-β-alanyl ryanodine (20mg, 0.035 mmol), DCC (20mg, 0.052 mmol) and DMAP (2mg, 0.018 mmol) in CH_2Cl_2 (10mL) at 0^0C. After 7 hr, 1mL of distilled water was added, stirring continued for an additional 10 minutes, and the solvents then removed under vacuum. The residue was redissolved in $CHCl_3$/2% MeOH and chromatographed on silica gel (10g). The product Az-βAR eluted with $CHCl_3$/6% MeOH and upon rotary evaporation and freeze-drying from dioxane, 6.1mg (30% yield), TLC R_f=0.29 ($CHCl_3$/MeOH/$MeNH_2$, 85:14.5:0.5) was obtained. (Non-radioactive V, IR 2100cm^{-1} (azide), *m/z* $(M+Li)^+$ 929.7, calcd. mol. wt. ($C_{38}N_6O_{13}H_{47}I$)=922.7. The specific activity of Az-βAR was 450mCi/mmol, (a total of 3.0mCi was incorporated into the product).

DISCUSSION

Over the last two decades photo-affinity labeling has become a valuable technique for localizing the binding domain(s) of ligands in

protein molecules (19-26). The heterobifunctional reagent, 4-azido salicylic acid N-hydroxysuccinimide ester (NHS-ASA) introduced by Ji and Ji (27) is especially useful, since in addition to being photoactivatable, it can be radio-labeled (more specifically, radio-iodinated), a characteristic that is desired of ligands for detecting the photo-affinity labeled complex.

Successful radio-iodination of NHS-ASA and subsequent condensation to ligands has been reported (27-28). However, we were unable to satisfactorily radio-iodinate this reagent and maintain integrity of the activated ester functionality. Three products, sometimes more, were observed. Thus, we turned our attention to preparing the synthon, N-(4-azido-salicyloyl)-β alanine, which can be coupled to ryanodine, dehydroryanodine, C_{10}-O_{eq}-glycyl ryanodine, C_{10}-O_{eq}-β-alanyl ryanodine or any one of the other amino or guanidino-acyl ester derivatives of ryanodine/dehydroryanodine previously described (16). In general, this synthon can be coupled to any ligand that contains an easily accessible sulfhydryl, alcohol (1^0 or 2^0) or amino (1^0) functionality using the method of Neises and Steglich (29).

Under the present reaction conditions, the preparation of the synthon N-(4-azido-5-^{125}iodo salicyloyl)-β alanine from 4-azido salicylic acid N-hydroxy succinimide ester proceeded to near completion; the yield of all the reactions being ≥92%. As such, a significant amount of the starting ^{125}I (ca 90%) is incorporated into the synthon. On the other hand, starting from 4-amino salicylic acid, 35% of the incorporated ^{125}I was lost during the formation of the azide function. Nonetheless, both 4-amino salicylic acid and 4-azido salicylic acid N-hydroxy succinimide ester were found useful as starting reagents for preparing the synthon, N-(4-azido-5-^{125}iodo salicyloyl)-β-alanine in acceptable yields. We were able to obtain at best a 60% yield for the condensation reaction of the synthon with C_{10}-O_{eq}-β-alanyl ryanodine.

Similar to [^{3}H] ryanodine, Az-βAR does not demonstrate saturation binding kinetics (Figure 2) (30). Non-radioactive Az-βAR (^{127}Az-βAR)

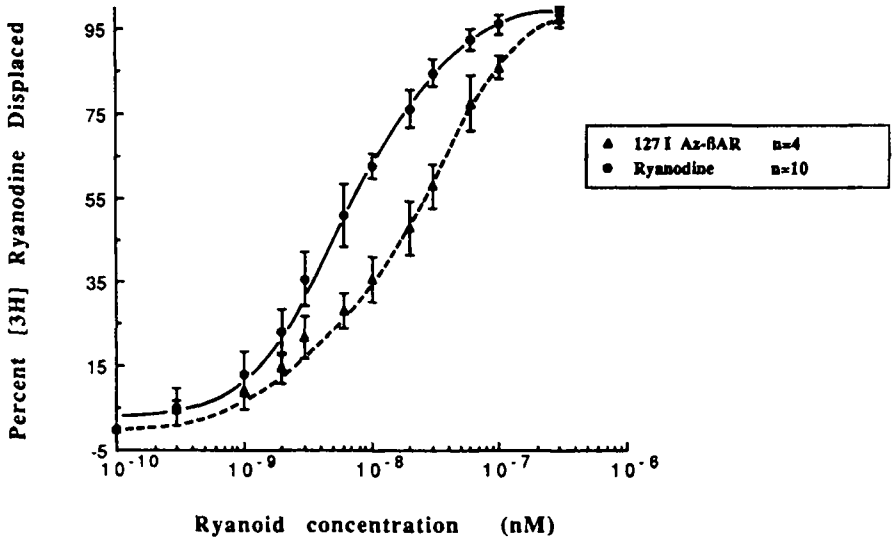


Fig. 2 Displacement of [^{3}H] ryanodine from the high affinity binding sites on the calcium-release channel/ryanodine receptor of skeletal muscle junctional sarcoplasmic reticulum vesicles by unlabeled alkaloid ryanodine and C_{10}-O_{eq}-N-(4-azido-5-^{127}iodo salicyloyl)-β- alanyl-β-alanyl ryanodine. Values displayed represent means ± SD for the number (n) of replicate determinations indicated, carried out on at least two different membrane preparations.

competed successfully with [^{3}H] ryanodine for binding to rabbit skeletal muscle ryanodine receptor. The IC_{50} was found to be 27.2 ± 2.0 x10^{-9} M (mean ± SD), and the K_D 8.8 ± 1.6x10^{-9} M (mean ± SD) (Figure 3). This relatively high affinity suggests that a significant amount of bulk can be accomodated in the binding domain where the C_{10} substituent resides, without adversely affecting binding. Evidence for such tolerance adjacent to the C_{10} position has been reported for N-carbobenzoyloxy β-alanyl ryanodine (16). It is therefore possible that the di-iodinated synthon N-(4-azido-2,5-^{125}diiodo salicyloyl)-β-alanine (readily formed by increasing the molar ratio of NaI), when coupled to ryanodine or β-alanyl ryanodine, may also result in a high affinity ryanodine derivative. If this

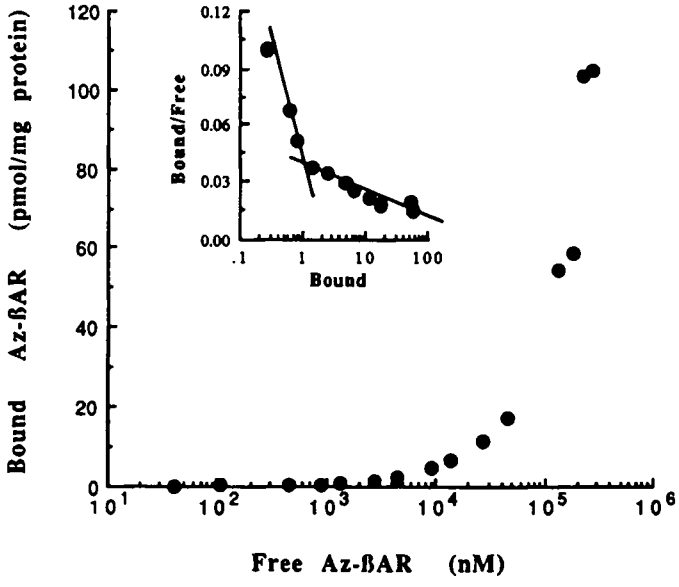


Fig. 3 Az-βAR binding to heavy SR vesicles using the procedure described by Humerickhouse et al. (31). Scatchard analysis (inset) reveals the presence of more than one binding site for Az-βAR.

is the case, then the synthesis of the diiodo synthon may be a simple, practical route for increasing the specific activity of this photo-affinity ligand.

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REFERENCES

(1) Jones, L. R., Besch, H. R. Jr., Sutko, J. L., and Willerson, J. T. J. Pharmacol. Exp. Ther. 209: 48 (1979).

(2) Sutko, J. L. and Willerson, J. T. Circ Res 46 332 (1980).

(3) Meissner, G. J. Biol. Chem. 261: 6300 (1986).

(4) Lattanzio, F. A., Schlatterer, M., Nicar, M., Campbell, K. P., and Sutko, J. L. J. Biol. Chem. 262: 2711 (1987).

5) Smith, J. S., Imagawa, T., Ma, J., Fill, M., Campbell, K. P., and Coranado, R. J. Gen. Physiol. 92: 1 (1988).

(6) Liu, Q. Y., Lai, F. A., Rousseau. E., Jones, R. V., and Meissner, G. Biophys. J. 55: 415 (1989).

(7) Fleisher, S., Ogunbunmi, E. M., Dixon, M.C. and Fleer, E. A. M. Proc. Natl. Acad. Sci. (USA) 82: 7256 (1985).

(8) Endo, M. Physiol. Rev. 57: 71 (1977).

(9) Stern, M. D., and Lakatta, E. G. FASEB J. 6: 3092 (1992).

(10) Galione, A. Science 259: 325 (1993).

(11) McPherson, P. S., and Campbell, K. P. J. Biol. Chem. 265: 18454 (1990).

(12) Walton, P. D., Airey, J. A., Sutko, J. L., Beck, C. F., Mignery, G. A., Sudhof, T. C., Deerinck, T. J., and Ellisman, M. H. J. Cell Biol. 113: 1145 (1991).

(13) Shosman-Barmatz, V., Pressley, T. A., Higham, S., and Kraus-Friedmann, N. Biochem. J. 276: 41 (1991).

(14) Staudermann, K. A., and Murawski, K. J. Biol. Chem. 266: 19150 (1991).

(15) Rardon, D. P., and Krause, P. C. In Frank, G. B., Bianchi, C. P., TerKeurs, H. E. D. (eds) Excitation-Contraction Coupling in Skeletal, Cardiac and Smooth Muscle. Adv. Exp. Med. Biol. 311: 405 (1992).

(16) Gerzon, K., Humerickhouse, R. A., Besch, H. R., Jr., Bidasee, K. R., Emmick, J. T., Roeske, R. W., Tian, Z., Ruest, L., and Sutko, J. L. J. Med. Chem. 36: 319 (1993)

(17) Mais, D. E., Burch, R. M., Saussy, D., L., Kochel, P. J., and Halushka, P. V. J. Pharmacol. Exp. Ther. 235: 729 (1985).

(18) Kiehem, D. J., and Ji, T. H. J. Biol. Chem. 252: 8524 (1977).

(19) Tometskom, M. A. and Richards, F. M (eds). Annals New York Aca. Sci. 346: (1980).

(20) Beneski, D. and Catterall, W. A. Proc. Natl. Acad. Sci. (USA) 77: 639 (1980).

(21) Denny, J. B. and Blobel, G. Proc. Natl. Acad. Sci. (USA) 81 5286 (1984).

(22) Shanahan, M. F., Wadzinski, B. F., Lowndes, J. M., and Ruoho, A., E. J. Biol. Chem. 260 10897 (1985).

(23) Safa, A. R., Hamel, E., and Felsted, R. L. Biochem. 26 97 (1987).

(24) Lowndes, J. M., Hokin-Neaverson, M. and Ruoho, A. E. Anal. Biochem. 168 39 (1988).

(25) Hylarides, M. D., Wilbur, S. D., Reed, M. W., Hadley, S. W., Schroeder, J. R. and Grant, L. M. Bioconjugate Chem. 2 433 (1991).

(26) Chatrenet, B., Kotzyba-Hibert, F., Mulle, C., Changeux, J. P., Goeldner, M. P. and Hirth, C. J. Pharmacol. Exp. Ther. 41: 1100 (1992).

(27) Ji, T. H. and Ji, I. Anal. Biochem. 121: 286 (1982).

(28) Myszka, O. G. and Swenson, R. P. Biochem. Biophys. Res. Comm. 172: 415 (1990).

(29) Neises, B. and Steglich, W. Angew. Chem. Int. Ed. (Eng) 117: 522 (1978).

(30) Meissner, G., Lai, A. F., Anderson, K., Xu, L., Lui, Q., Herrmann-Frank, A., Rousseau, E., Jones, R. V., and Lee, H. In Moreland, R. S (ed) Regulation of Smooth Muscle Contraction. Plenum Press, New York. 241 (1991).

(31) Humerickhouse, R.A., Besch, H.R. Jr., Emmick, J.T., Gerzon, K., Ruest, L. and Sutko, J.L. Mol. Pharmacol. (In Press 1993).