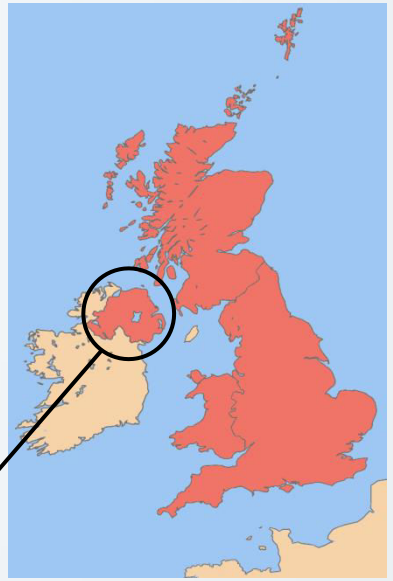
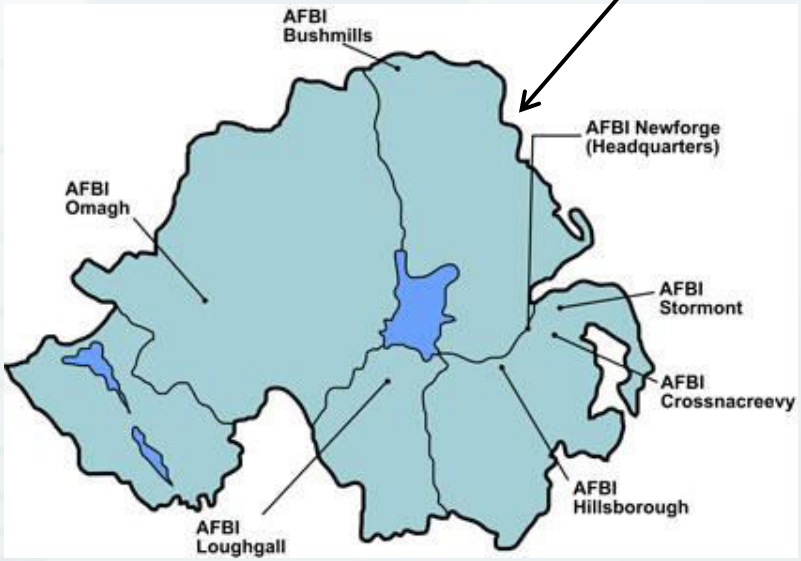
# Production of antisera to phenylbutazone and oxyphenylbutazone for use in immunochemical detection assays

Terry Fodey,
Chemical Surveillance Branch,
Veterinary Sciences Division,
Agri-Food and Biosciences Institute, Belfast, UK

# AFBI Location and Work Areas

- Animal Health and Welfare
- Crops and Grassland
- Economics and Rural Development
- Environment and Land-use
- Fisheries and Aquatic Ecosystems
- **Food**
- Livestock






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# AFBI – Stormont, Veterinary Sciences Division

- Virology
- Bacteriology
- Disease Surveillance and Investigation



- **Chemical Surveillance**
  - National Reference Laboratory for veterinary drug residue control
  - Immunological screening assays - antibody production
  - Microbiological and TLC screening assays
  - Chromatography and mass spectroscopy chemical confirmation methods
    - Monitoring chemical residues in meat, milk, eggs and shellfish

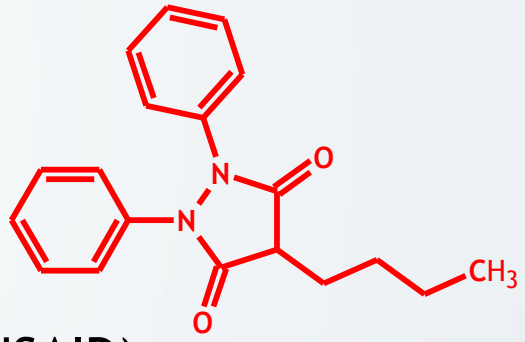
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# Scope of presentation

- What is Phenylbutazone?
- Why do we need an antibody to it?
- Approaches to antibody production
- Comparison of antisera performance
- Conclusions

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# What is Phenylbutazone?




- A non-steroidal anti-inflammatory drug (NSAID)
  - Abbreviated to PBZ; often called “Bute”
- Introduced to human medicine in 1949 for the treatment of rheumatoid and arthritic disorders
- Found to induce disorders of the blood including aplastic anaemia, leukopenia, granulocytosis and thrombocytopenia, in some cases leading to death
- Consequently, its use in human medicine became limited with the licence for use in man revoked in the U.K. in 1984

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# Use in horses

- Choice of NSAID for equines since its introduction to veterinary medicine in the 1950s
- Substantial clinical history of efficacy and safety in horses accumulated over both short and long treatment periods
- Horse meat is consumed in many countries
- The CVMP assessed PBZ in 1997:
  - Health risks are blood dyscrasias and the genotoxic & carcinogenic potential
  - No thresholds identified so maximum residue limits could not be established
  - Therefore PBZ is not permitted for use in any food producing animals

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# Eu protocols to prevent PBZ treated horses entering food chain

- Since 2005 horses must have a passport for identification
  - Must be signed to declare if the animal is intended for human consumption
  - However PBZ prescribed without checking passport
  - Subsequent failure to sign out of food chain after prescription
  - Horses change owners with passport not signed to exclude from food chain
  - Medicated meal for one horse may be consumed by another animal

(VMD)

*VMD (Veterinary Medicines Directorate)*

*http://aka-vmd.defra.gov.uk/VRC/pdf/PositionPaper_Phenylbutazone.pdf*

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# PBZ detection in Eu

- From 2008-2012, 5.4% of equines tested were non-compliant
- From 2008-2012, 0.3% of bovines tested were non-compliant

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# European Commission Press Release

## Brussels, 12 September 2014

EUROPEAN COMMISSION

PRESS RELEASE

Brussels, 12 September 2014

### Animal Health: EU to tighten rules on horse passports

A Commission proposal to revise the rules for the identification of horses was endorsed yesterday by EU Member States' experts[1]. This revised Regulation provides a more reliable and safer European system for the registration and identification of horses in the EU. One of the basic aims is to prevent the inadvertent or fraudulent slaughter for human consumption of horses which must be excluded from the food chain.

EU Commissioner in charge of Health, Tonio Borg said: "As promised, this is another lesson drawn from last year's horse meat fraud: the rules endorsed by the member States will strengthen the horse passport system in place. I believe that closer cooperation will enhance the safeguards which prevent non-food quality horse meat from ending up on our plates".

### Main elements

With nearly 7 million equidae in Europe, the revised rules will require foals to be issued with a single passport having a unique identification number before their first birthday

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# European Commission Press Release

## Brussels, 12 September 2014

**Main elements**

With nearly 7 million equidae in Europe, the revised rules will require foals to be issued with a single passport having a unique identification number, before their first birthday. The passport also serves as a medical record and will serve the horse over its lifetime. All horses born after 1 July 2009 will need to be micro-chipped. Technical security features aimed at reducing the risk of falsified passports have also been put in place.

The introduction of a compulsory centralised database in all Member States will assist the competent authorities to better control the issuance of the passports by different passport issuing bodies. It will also substantially simplify, for the keepers, the procedures for updating the identification data in both the passport and the database of the issuing bodies.

**Next steps**

The Regulation will apply from 1 January 2016. However, EU countries not already having a centralised database will have until 1 July 2016 to put one in place.

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# Residue sources

- Feeding vessels can be contaminated
- Untreated animals can become contaminated from treated ones
- Fraudulent use of horsemeat as a substitute for beef
- Uninformed use of drug
- Illegal use of drug

Fodey, T. L., Smyth, W.G., Barnes, P., Traynor, I.M., Kennedy, D.G., Crooks, S.R.H. (2015) Investigation into sources of contamination of cattle with phenylbutazone. *Veterinary Record* 176(3) 74-76

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# Contamination Investigation

Downloaded from http://veterinaryrecord.bmj.com/ on April 23, 2015 - Published by group.bmj.com



## Short Communication

### Investigation into sources of contamination of cattle with phenylbutazone

Terence L. Fodey, Wesley G. Smyth, Paul Barnes, Imelda M. Traynor, D. Glenn Kennedy, Steven R. H. Crooks

PHENYLBUTAZONE (PBZ), also known as 'bute', is a NSAID authorised to treat horses suffering from musculoskeletal disorders such as rheumatoid and arthritic diseases and to relieve them from the associated pain.

An assessment of PBZ by The Committee for Medicinal Products for Veterinary Use (CVMP) (European Medicines Agency 1997) found that the main health risks to the consumer were blood dyscrasias and the genotoxic/carcinogenic potential for which no thresholds could be identified and so no maximum residue limits could be established. As a consequence of this assessment, PBZ is not permitted for use in any animal destined for the food chain. The risks were reconfirmed more recently in a joint statement by the European Food Safety Authority and surrogates for legally treated horses in the scenarios described, which could lead to contamination of other cattle.

Pro-Dynam oral powder, manufactured by Dechra Veterinary Products A/S (Denmark), was used to deliver the drug to the treated animals. Each 5 g sachet contains 1 g of PBZ along with the excipients glucose monohydrate and methylhydroxypropylcellulose. The dosage scheme recommended for a 450 kg horse was followed for the cattle of similar weight:

Day 1: two sachets twice a day (4.4 mg/kg on each occasion)
Days 2–4: one sachet twice a day (2.2 mg/kg on each occasion)
Day 5: one sachet

The powder was sprinkled on approximately 500 g of a coarse cattle ration in a feeding bucket and given to a bullock (T1) which consumed the contents within five minutes. After each occasion of drug administration, 500 g of cattle ration was placed in the same bucket without cleaning and given to a second bullock (B1) housed in a separate pen. Blood samples (10 ml) were taken from B1 twice a week. A third bullock (T2) was treated with PBZ in the same way as T1. When treatment was completed, T1 was moved into a different house containing three other bullocks (H1, H2 and H3). Blood samples were collected from these three animals on a daily basis for a week and then twice a week as before. After treatment, T2 was moved into a paddock (approximately 30×12 m) for four days. Once it was removed, a second group of three bullocks (P1, P2 and P3) was placed in the paddock and allowed to graze for three days. Blood samples were collected from these three animals on a daily basis for a week and then twice a week after that. Twenty days after these animals were removed, three bullocks (F1, F2 and F3)

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# PBZ detection in Eu

- From 2008-2012, 5.4% of equines tested were non-compliant
- From 2008-2012, 0.3% of bovines tested were non-compliant
- Physicochemical methods of analysis exist
- Community reference laboratory recommended detection:

| Substance | Matrix | Recommended conc |
|---|---|---|
| Phenylbutazone<br>Oxyphenylbutazone | Muscle, kidney, liver, milk, plasma | 5 ppb |

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# Metabolites and analogues

- The principal metabolites are oxyphenylbutazone and γ-hydroxyphenylbutazone
- They possess analgesic/anti-inflammatory properties and thus contribute (probably small-moderate degree) to the pharmacological actions of phenylbutazone
- For both metabolites, urine concentrations are much higher than those in plasma
- Suxibuzone is a pro-drug of PBZ, designed to reduce gastro-intestinal disturbances. Also banned in food producing animals

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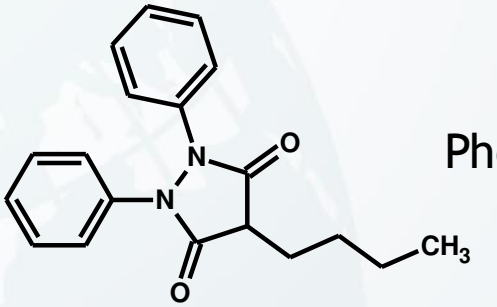
# Immunogenicity

- PBZ and its analogues are low molecular weight ranging from 308.4 Da (PBZ) to 428.5 Da (SBZ)

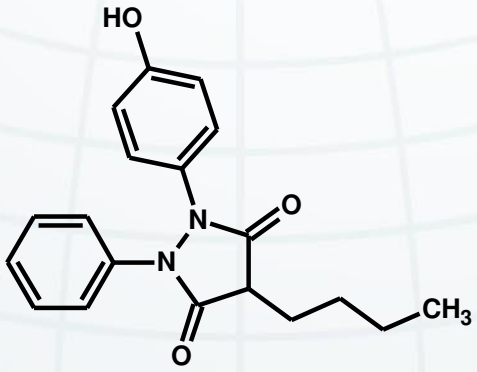
- They need to be conjugated to large carrier proteins to render them immunogenic

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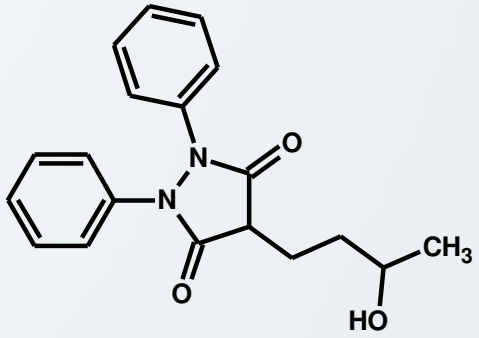
# Hapten options for antibody production



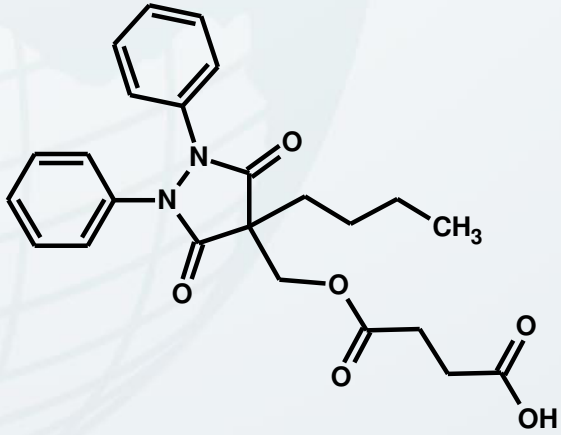

Phenylbutazone




Oxyphenylbutazone



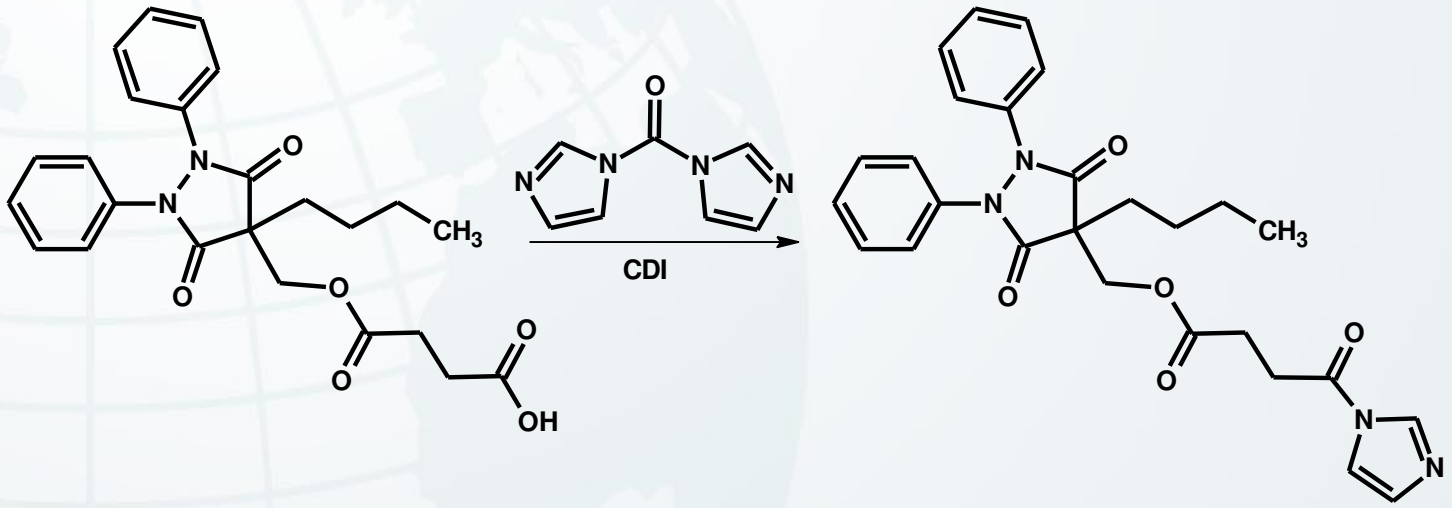

γ-Hydroxyphenylbutazone




Suxibuzone

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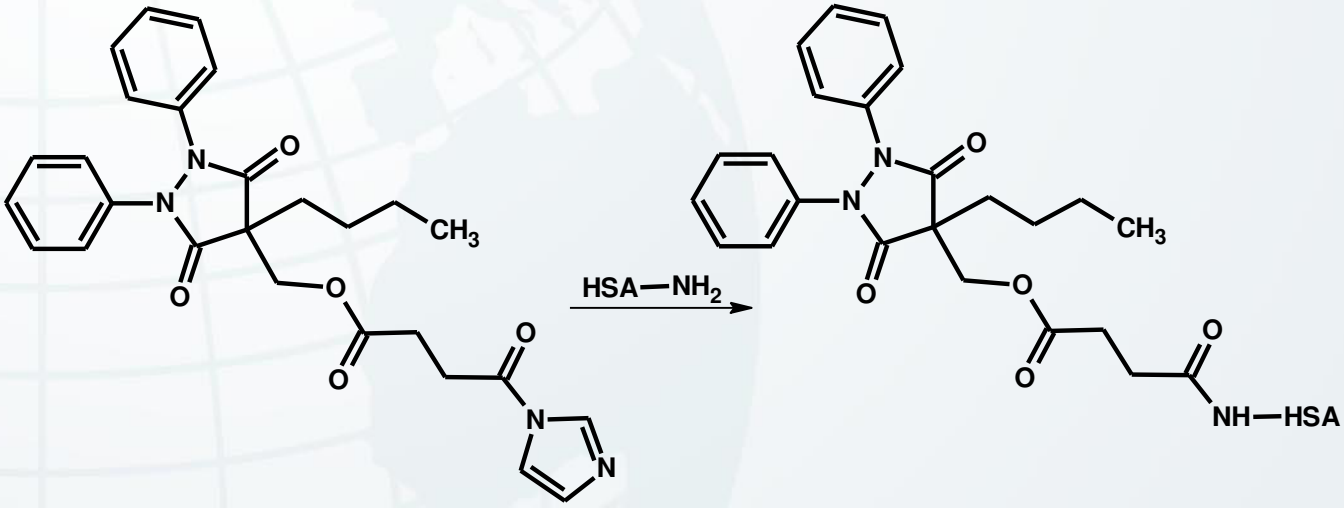
# Suxibuzone-HSA (step 1)




Carboxylic acid activation via 1,1'-carbonyldiimidazole creates acyl imidazole reactive intermediate

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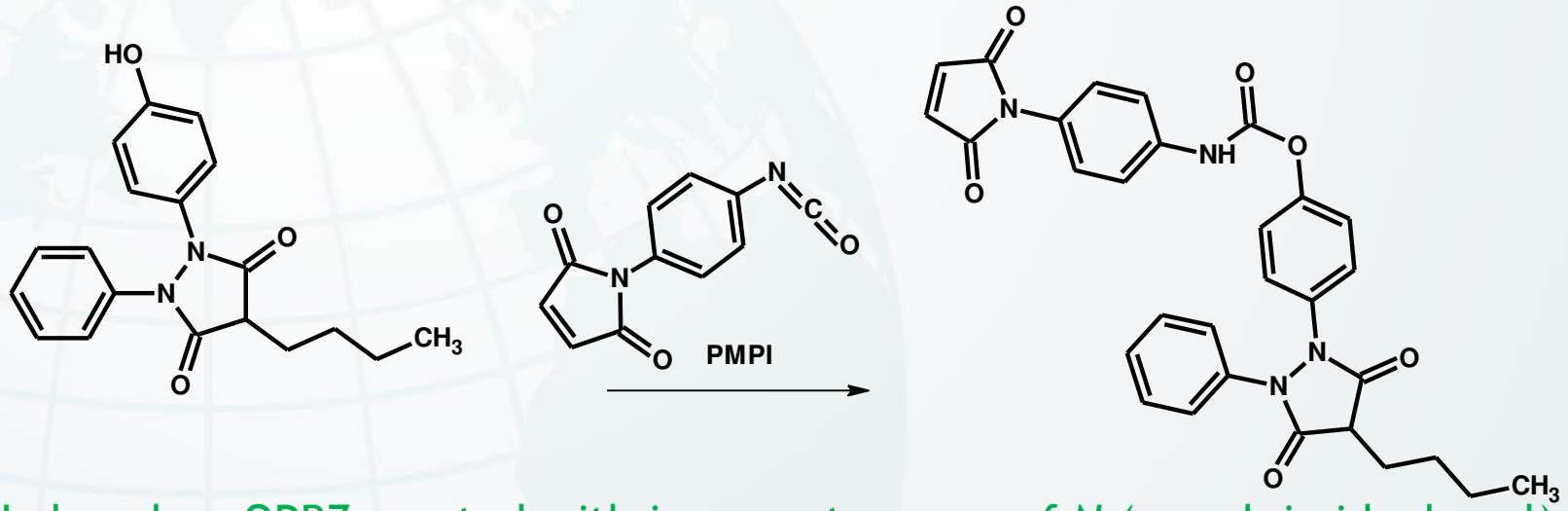
# Suxibuzone-HSA (step 2)



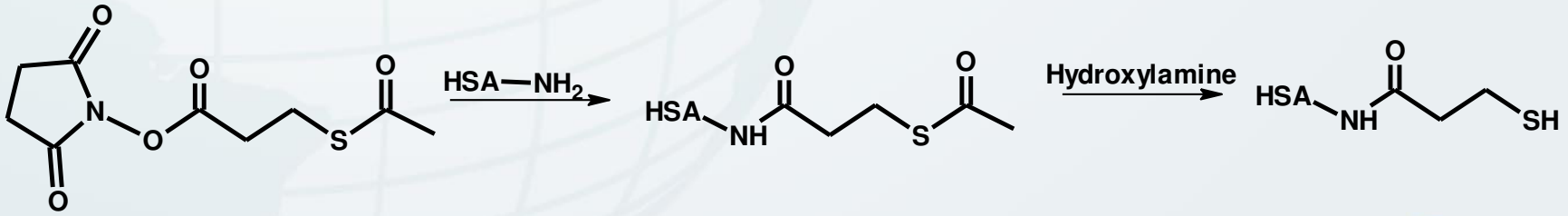

Acyl imidazole intermediate species reacts with primary amine on carrier to generate stable amide linkage

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# Oxyphenylbutazone-HSA (step 1)



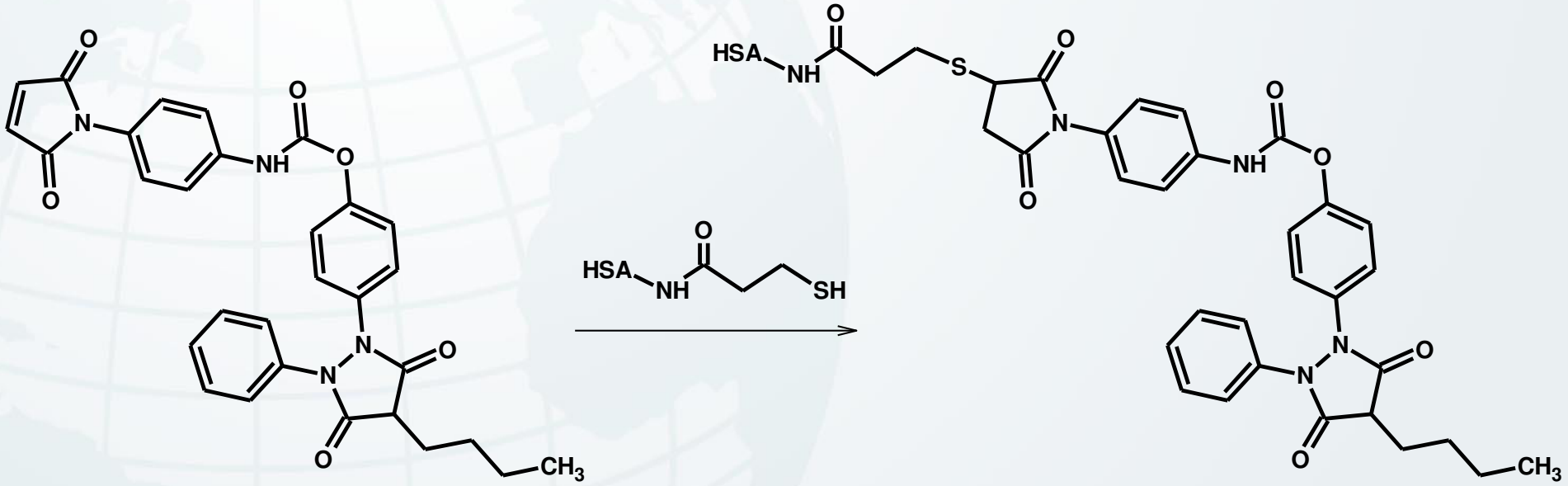

Hydroxyl on OPBZ reacted with isocyanate group of *N*-(*p*-maleimidophenyl)-isocyanate (PMPI) leaving maleimide group available for reaction




N-succinimidyl-S-acetylthiopropionate (SATP) reacted with primary amine on carrier to add a sulfhydryl group for reaction

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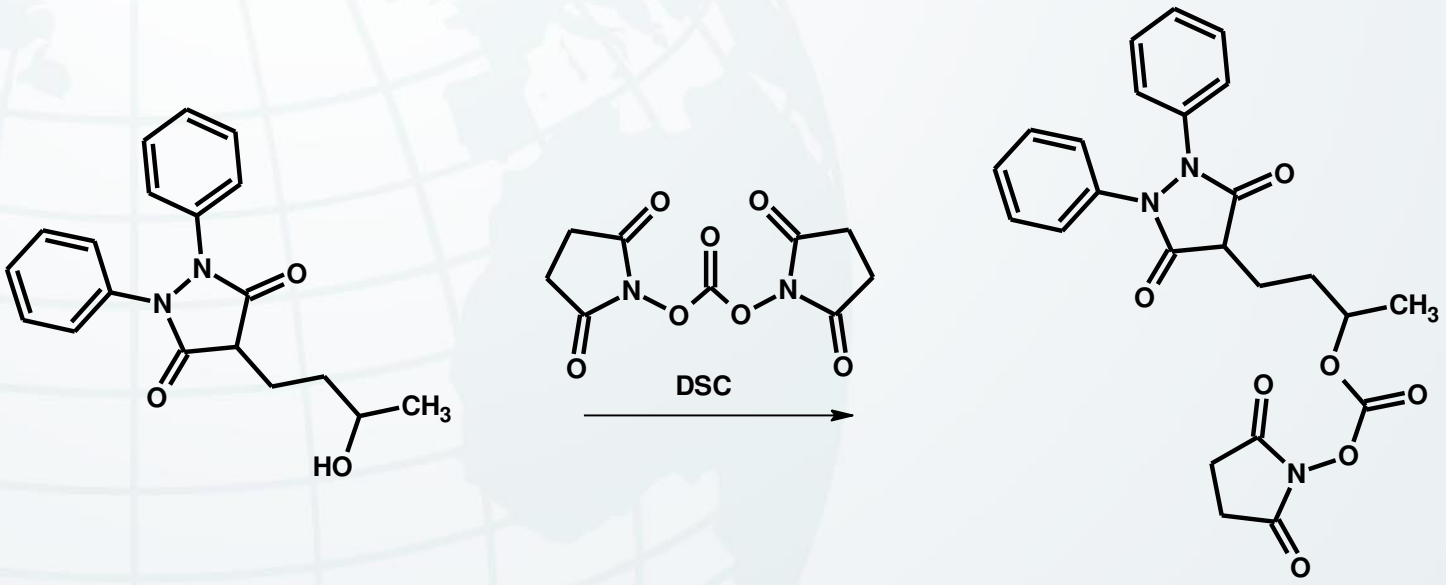
# Oxyphenylbutazone-HSA (step 2)




Maleimide and sulfhydryl groups brought together to create coupling of hapten to carrier

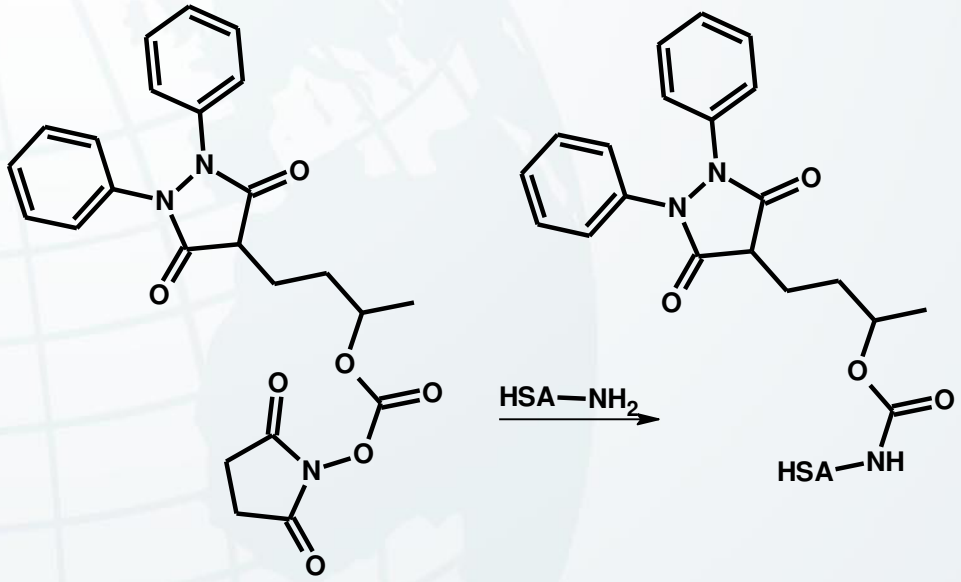
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# γ-Hydroxyphenylbutazone-HSA (step 1)




Hydroxyl group on HPBZ is activated with N,N'-Disuccinimidyl carbonate to create succinimidyl carbonate intermediate

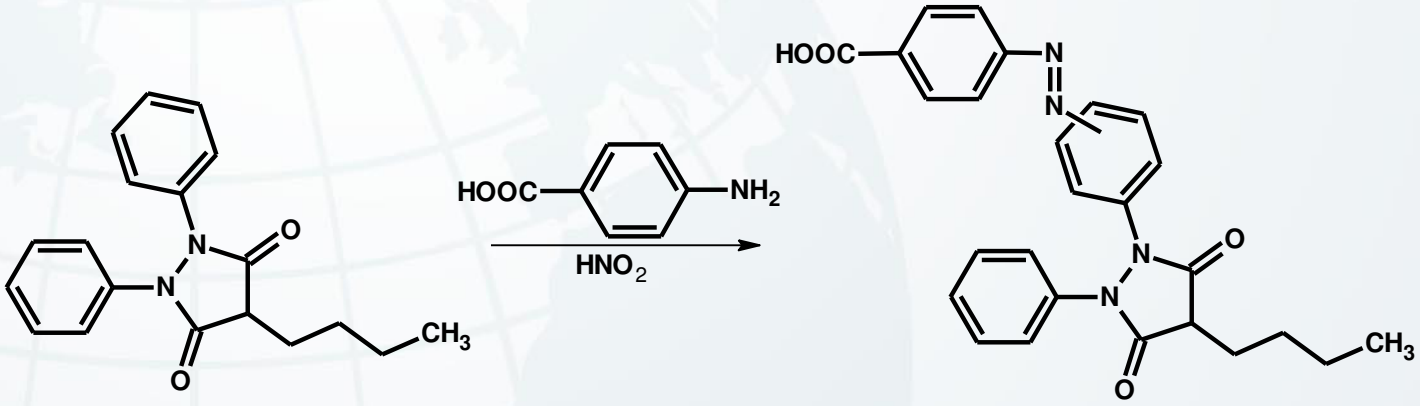
# γ-Hydroxyphenylbutazone-HSA (step 2)




Succinimidyl carbonate intermediate is reacted with amine on carrier to produce carbamate linkage

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# Phenylbutazone-HSA (step 1)

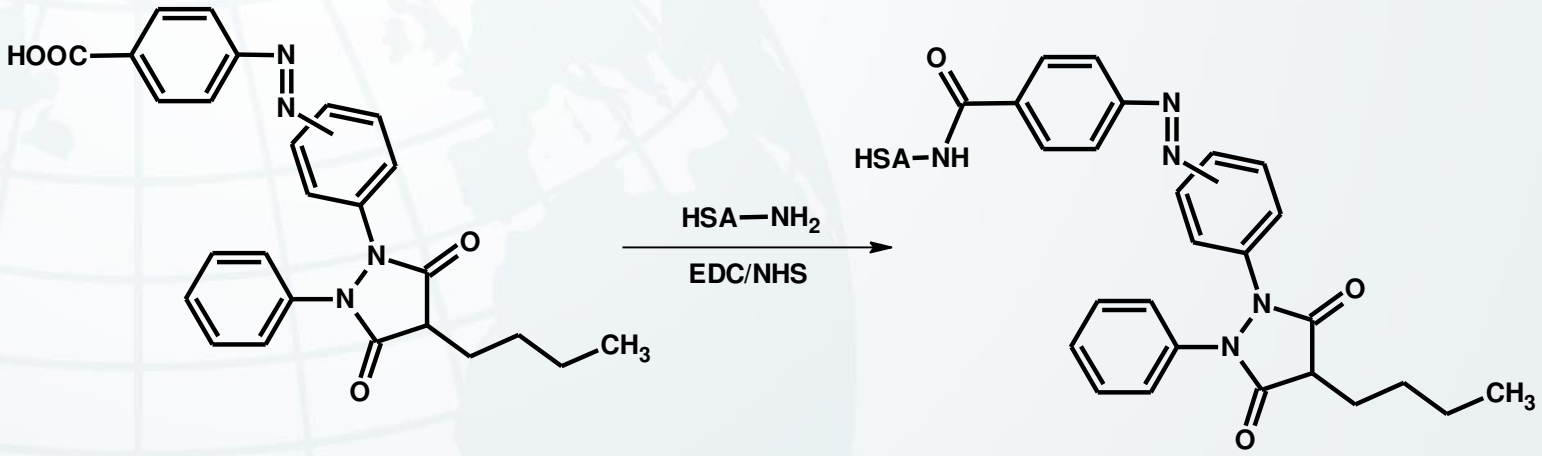



*p*-Amino benzoic acid is diazotised before adding to PBZ

Kawashima, K., R. Dixon and S. Spector (1975) Development of radioimmunoassay for chlorpromazine. *European Journal of Pharmacology* 32(2)195-202

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# Phenylbutazone-HSA (step 2)




The newly formed carboxylic acid is activated by carbodiimde reaction to allow coupling to carrier via amine

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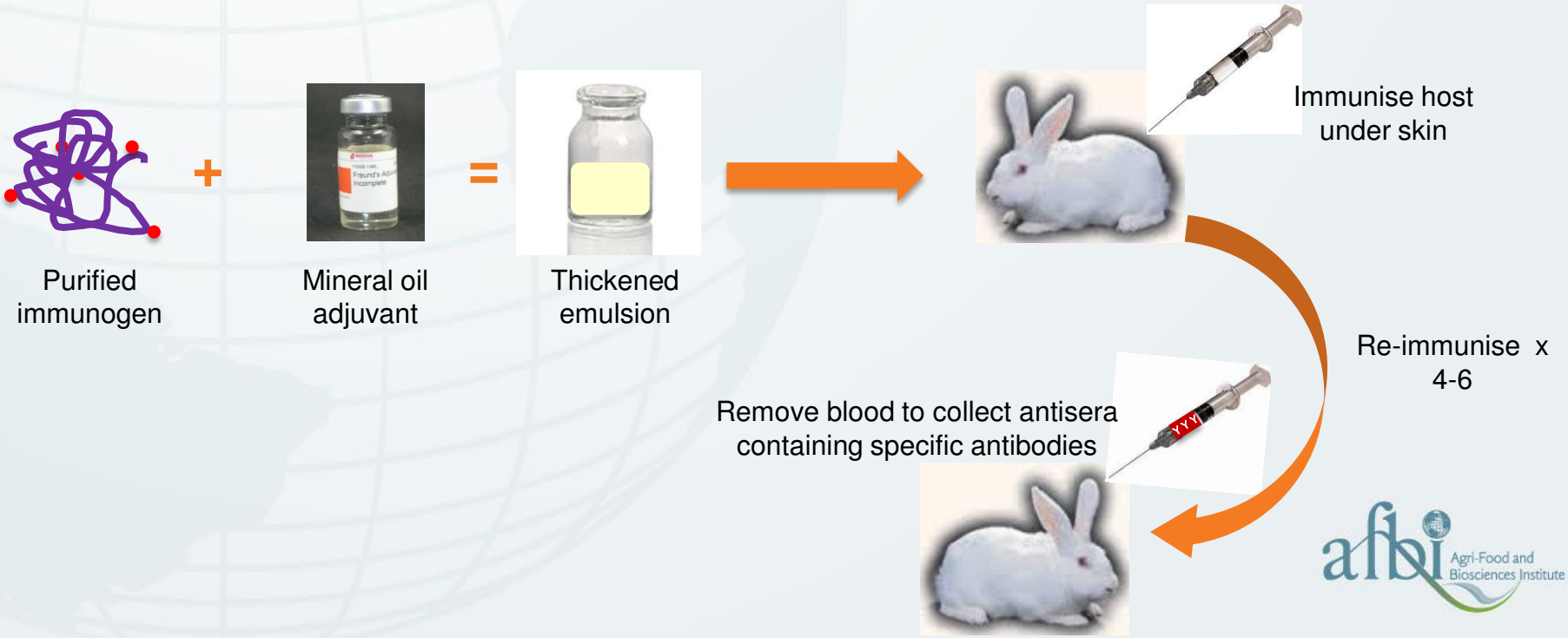
# Enzyme labels

- Horseradish peroxidase was conjugated to each hapten by the same method as for the immunogen production
- Each of the 4 enzyme labels were used for determination of antibody titre in direct competitive ELISA checkerboards for all sera samples
- This created homologous and heterologous assay formats

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# Immunisation

- After conjugation the immunogens were purified and mixed with an adjuvant before administration to the host
- Two rabbits were immunised for each immunogen



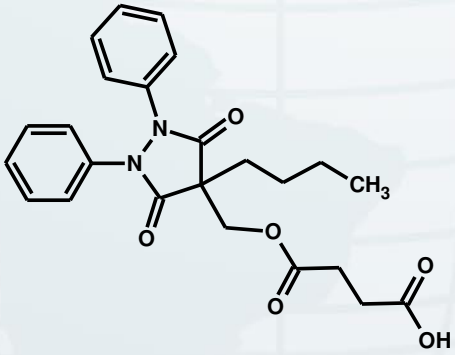
# Immunisation

- First boost after two weeks, then monthly
- Blood samples taken 10 days after each immunisation were assessed for specific antibodies
- Number of immunisations ranged from 6 to 8 before a satisfactory titre was obtained

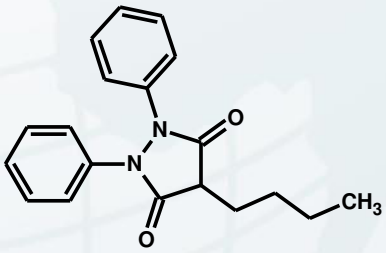
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# Homologous vs Heterologous

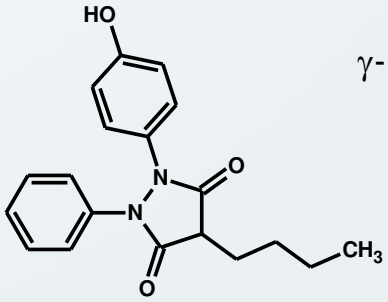
- The SBZ-HRP enzyme label performed better than the other HRPs in combination with all antisera
- Therefore for PBZ, OPBZ and HPBZ assays were heterologous while SBZ assay was homologous



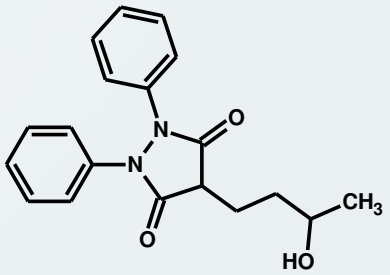
Suxibuzone



Phenylbutazone



Oxyphenylbutazone



γ-Hydroxyphenylbutazone

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# Antisera sensitivity and specificity

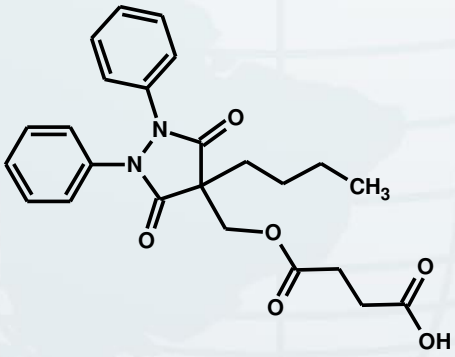
| | $IC_{50}$s (ng/ml) [% cross-reactivity] | | | |
|---|---|---|---|---|
| **Immunogen** | **PBZ** | **OPBZ** | **HPBZ** | **SBZ** |
| **PBZ-HSA (1)** | 18.3 [100] | 53.9 [34] | >100 [ND] | 94.6 [19] |
| **PBZ-HSA (2)** | 5.5 [100] | 57.2 ]10] | >100 [ND] | 5.5 [100] |
| **SBZ-HSA (1)** | 4.6 [198] | 89.2 [10] | >100 [ND] | 9.1 [100] |
| **SBZ-HSA (2)** | 3.6 [39] | >100 [ND] | >100 [ND] | 1.4 [100] |
| **OPBZ-HSA (1)** | 5.8 [97] | 5.6 [100] | >100 [ND] | 2.2 [255] |
| **OPBZ-HSA (2)** | >100 [ND] | >100 [ND] | >100 [ND] | >100 [ND] |
| **γ-HPBZ-HSA (1)** | 7.7 [165] | >100 [ND] | 12.7 [100] | 7.3 [174] |
| **γ-HPBZ-HSA (2)** | 0.9 [433] | 9.3 [42] | 3.9 [100] | 3.7 [105] |

ND = Not determined
All data produced using SBZ-HRP in direct competitive ELISAs

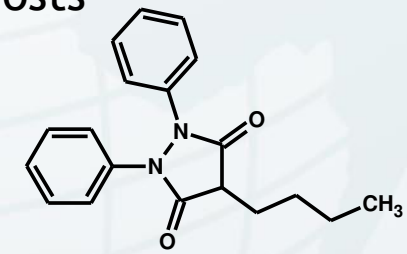
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# Conclusions

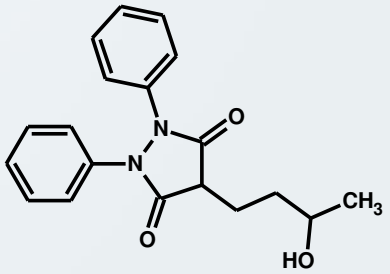
- Antisera to PBZ can be produced from all four haptens with γ-HPBZ and SBZ providing the best sensitivity
- The most sensitive antisera to OPBZ are produced from OPBZ hapten
- Only γ-HPBZ immunogen produced antisera capable of binding γ-HPBZ
- As has been previously established there are significant variations in immune response between hosts



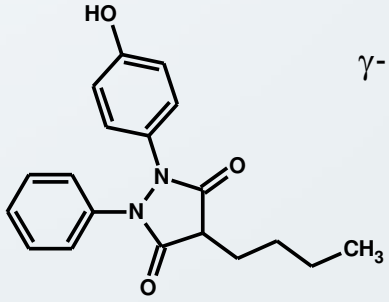
Suxibuzone



Phenylbutazone



γ-Hydroxyphenylbutazone



Oxyphenylbutazone

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# Thanks


- Colleagues from AFBI: Mr Paul Barnes and Dr. Steven Crooks for their assistance in this study

- Professors Smith and Eremin for hosting this symposium and providing the opportunity for me to present this work


- You for your attention

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