Molecular and Biochemical Mechanisms of Detoxification of Aflatoxin and Mycotoxins in Navel Orangeworm

Project No.: **08-ENTO1-Berenbaum**

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Objectives:

Mycotoxins, such as aflatoxins and ochratoxins, are widely distributed in nature and are frequently encountered by herbivores (Park, 2002). Mycotoxins are responsible for the annual loss of millions of dollars in the United States due to crop contamination. Insect infestation significantly exacerbates aflatoxin contamination of crops; insect control not only reduces direct feeding damage but also reduces aflatoxin contamination (Dowd, 2001; Schatzki and Ong, 2000). The goal of this project is to determine mechanisms underlying interactions between fungi and plant-feeding insects in nature and obtain knowledge that can be applied toward developing practical methods to decrease mycotoxin contamination in the nut industry. We specifically focus on the Navel Orangeworm (NOW) *Amyelois transitella* (Walker) (Lepidoptera: Pyralidae), a serious pest of almonds, walnuts and pistachios, with five objectives:

1. To determine the toxicity of mycotoxins (aflatoxin, ochratoxin and others) to Navel Orangeworm (NOW);
2. To study the synergistic effects of synthetic and natural compounds on toxicity of mycotoxins to NOW;
3. To identify the enzymes involved in detoxification of mycotoxins;
4. To determine the influence of host plants on the detoxification of mycotoxins by examining effects of host plant allelochemicals on the toxicity of mycotoxins in strains of NOW originating from different hosts;
5. To examine effects of fungi on the development and survival of larvae of NOW

Interpretive Summary:

I. Toxicity of aflatoxins and ochratoxins to NOW (*A. transitella)*

The toxicity of aflatoxin B1 and ochratoxin A to *A. transitella* has been evaluated at different developmental stages. The results show that *A. transitella* can tolerate very high levels of AFB1 in its diet. The concentration of 1 µg/g AFB1 has no toxic effects on 1st instar *A. transitella,* nor did AFB1 at higher concentration (10 and 20 µg/g). After eighteen days, the pupation rate was 76% and 78% on the diets containing 10 µg/g and 20 µg/g AFB1, respectively (Table 1). At extremely high concentrations, AFB1 (100 µg/g) strongly inhibited the development of *A. transitella* at first instar and caused 30% mortality in seven days. Even at this extremely high concentration of AFB1, 30% iof larvae survived at least 3 weeks. The ability of *A. transitella* to tolerate AFB1 increased over the course of development. Ultimate instar larvae tolerated 100 µg/g AFB1 with only a slight decrease in pupation rate compared with insects feeding on control diets containing 0.5% percent DMSO.

The toxicity of AFB1 to *A. transitella* was compared with its toxicity to *Helicoverpa zea*, a lepidopteran that encounters aflatoxins less frequently in its host plants. From Fig. 2, we can see that the LC_{50} (defined as the concentration of AFB1 that causes 50% of newly hatched larvae to fail to enter second instar in 48 hours) of AFB1 to *A. transitella* is 100 times greater than the LC_{50} of AFB1 to *H. zea*. In addition, *A. transitella* can survive on diets containing much higher concentrations of aflatoxins than *H. zea*. Whereas AFB1 at a concentration of 1 µg/g AFB1 caused 100% mortality for both 1st instar and 3rd instar larvae of *H. zea*, no significant toxic effects were observed on *A. transitella* 1st or 4th instars at this concentration (Zeng et al., 2006).

The ability to tolerate high concentrations of aflatoxins makes *A. transitella* a convenient vector for spreading mycotoxin-releasing fungi in orchards. Navel Orangeworm is a serious pest of the nut industry not only because of the damage it can cause directly to fruits but also because of the indirect damage it can cause by increasing concentrations of mycotoxin in nut products and reducing their value.

II. Metabolism of aflatoxins by NOW (*A. transitella*)

To investigate the ability of *A. transitella* to turn over aflatoxins, we conducted metabolism experiments with midgut proteins. Midguts were dissected from 40 final instar larvae and stored at -80 ºC. The frozen midguts were ground to a fine powder using mortars and pestles in the presence of liquid N_2 and then 1ml M phosphate buffer was added (pH 7.5). The proteins were isolated by centrifuging the homogenate. AFB1 metabolism assays were conducted with the proteins in 250 µl reaction system and initiated with the addition of NADPH. Two polar metabolites (Met1 with retention time of 7.8 min and Met2 with retention time of 8.0 min) were readily detected (Fig. 3B). No extra peak was detected when mouse liver cytosol, an AFBO trapping agent, was added into the reaction, which indicates that AFB1 is transformed only into detoxified (in contrast with bioactivated) metabolites by the midgut proteins. The metabolites will be identified with LC-MS in future studies. Compared with another lepidopteran, *H. zea*,

the midguts of *A. transitella* larvae have higher turnover rates than *H. zea* (32 pmol/min/mg vs 0 pmole/min/mg).

III. Identification of P450s involved in detoxification of aflatoxins from NOW (*A.transitella)*

A.transitella has an efficient defense system for processing remarkably high concentrations of aflatoxins and other mycotoxins in its food. Cytochrome P450s as phase I detoxification enzymes play an important role in detoxification of mycotoxins (Eaton, 1995; Feyereisen, 1999). In view of their extraordinary activity, P450 enzymes from *A. transitella* may be have potential industrial applications if bioengineered to process and eliminate aflatoxins in seriously contaminated nuts or other crops in the future.

Three full-length P450 cDNAs were identified from midguts of larvae of *A. transitella* and named CYP321C1, CYP6B44 and CYP6AB11 based on sequence. The amino acid alignment of these P450 genes in Fig. 4 shows that these three protein sequences have quite variable amino acid substitutions in substrate recognition sites (SRS), suggesting that substrate specificity may vary among these enzymes. All three genes have been expressed in the Bac-to-Bac baculovirus expression system to characterize their catalytic activity toward mycotoxins and host plant allelochemicals**.**

IV. To study the synergistic effects of synthetic and natural compounds on toxicity of mycotoxins to NOW (*A. transitella)*

As has been demonstrated in other lepidopterans, synthetic as well as naturally occurring host plant compounds have the potential to synergize toxicity of mycotoxins in *A. transitella*. Piperonyl butoxide (PBO) as a general inhibitor of P450s has been applied in combination with various insecticides to increase mortality and decrease longevity in response to pesticides. In this study, there was no significant difference in growth and development of larvae feeding on AFB1 in the presence of PBO and the insects feeding on AFB1 alone (data not shown). We are in the process of evaluating the synergistic potential of naturally compounds from essential oil such as apiol or myristicin. Studies of mycotoxin synergists can provide insight into the mechanism of detoxification of mycotoxins by insects and also can provide the basis for developing synergists as specific inhibitors to deal with *A. transitlella* in orchards.

V. The effects of fungus on the survival of NOW (*A. transitella)*

Mycotoxin-releasing fungi may be very important for survival of *A. transitella* in nature. The fungi can provide a suitable microenvironment for the insects to survival by maintaining stable humidity and temperature or by protecting insects from their natural enemies via association with fungal toxins. Our preliminary data revealed that insects feeding on dry almonds grew much more slowly than the insects feeding on artificial diet which is rich in nutrition. It is very interesting that the insects still grew and developed very well when we transferred them to fungi-contaminated artificial diets. A series of

bioassay have been set up to determine the effects of the fungi on the development and growth rate of Navel Orangeworms.

VI. The effects of plant allelochemicals from host plants on the detoxification of mycotoxins by strains of *Amyelois transitella* originating from different host plants

Plants produce a wide range of secondary compounds (allelochemicals) to protect them from herbivores and pathogens (Berenbaum, 1985). Thus, insect herbivores have to tolerate a wide range of chemically diverse plant toxins in order to utilize these plants as hosts (Li et al., 2002b). We have recently demonstrated that insects such as *Helicoverpa zea* can utilize host allelochemicals to induce the activity of the detoxification enzymes involved in metabolizing mycotoxins (Zeng et al., 2007). So it is of interest to determine whether the kernel-eating specialist, *A. transitella*, can utilize its host plant chemistry to defend against mycotoxins.

The ongoing research is focusing on xanthotoxin, a furanoucoumarin, which has been found in fruits and leaves in figs, a NOW host. We are conducting bioassays to compare the toxicity of xanthotoxin to the strain collected from figs in the field with the laboratory strains originating from almonds or from pistachio.

References

Berenbaum MR, 1985. Brementown revisited: Interactions among allelochemicals in plants. Recent Adv. Phytochem. 19:139–169.

Dowd PF, 2001. Biotic and abiotic factors limiting efficacy of Bt corn in indirectly reducing mycotoxin levels in commercial fields. J. Econ. Entomol. 94:1067-1074.

Eaton LD, Gallagher PE, 1994. Mechanisms of aflatoxin carcinogenesis. Annu. Rev. Pharmacol. Toxicol. 34:135-172.

Feyereisen R, 1999. Insect P450 enzymes. Annu. Rev. Entomol. 44:507-533.

Li X, Schuler MA, Berenbaum MR, 2002b. Plant allelochemicals differentially regulate *Helicoverpa zea* cytochrome P450 genes. Insect Mol. Biol. 11:343-351.

Park DL, Troxell TC, 2002. U.S. perspective on mycotoxin regulatory issues. Adv. Exp. Med. Biol. 504:277-285.

Schatzki TF, Ong MS, 2000. Distribution of aflatoxin in almonds. 2. Distribution in almonds with heavy insect damage. J. Agric. Food Chem. 48:489-492.

Zeng RS, Niu G, Wen Z, Schuler MA, Berenbaum MR, 2006. Toxicity of aflatoxin B1 to *Helicoverpa zea* and bioactivation by cytochrome P450 monooxygenases. J. Chem. Ecol. 32:1459-1471.

Zeng RS, Wen Z, Niu G, Schuler MA, Berenbaum MR, 2007. Allelochemical induction of cytochrome P450 monooxygenases and amelioration of xenobiotic toxicity in *Helicoverpa zea*. J Chem Ecol. 2007 33(3):449-61.

Legends:

Table 1
Pupation rate and pupal weight of NOW (*A. transitella*) larvae after exposure to different concentrations of aflatoxin B1 in diet at first instar. Values for pupation rate are means +standard errors from 20 caterpillars/treatment with three experimental replicates for each. Values for pupal weight are representative of one of the three series of replicates with the number of insects that have pupated for this replicate shown above each bar.

Figure 1
Developmental effects of aflatoxin B1 on 1st instar larvae of *H. zea* and NOW (*A. transitella).* (A) Percentage of *H. zea* larvae that have molted to second instar after 48 hours of treatments with different concentrations of AFB1. (B) Percentage of *A. transitella* larvae that have molted to second instar after 48 hours of treatments with different concentrations of AFB1.

Figure 2
HPLC chromatogram showing the metabolism of AFB1 by midgut proteins of final instar larvae of NOW (*A. transitella)* to form two metabolites which were eluted at 7.8 and 8.0 min, respectively (Fig.2B) compared with control reaction (Fig.2A). AFG1 (retention time 10 min) represents the internal standard used for normalization of AFB1 remaining in each sample.

Figure 3
Alignment of amino acid sequences of P450s from NOW (*A. transitella)* and that from *H. zea*. CYP321A1 and CYP6B8 were identified from *H. zea*; CYP6321C1, CYP6B44 and CYP321C1 were identified from NOW (*A. transitella)*. SRS means substrate recognition sites. The meanings of the consensus key are as following: "*" represents single, fully conserved residue; ":" represents conservation of strong groups; "." represents conservation of weak groups; "no label" represents no consensus.

Table 1. Toxicity of Aflatoxin B1 to 1st instar of NOW (*A. transitella)*

Concentrations of AFB1	**Pupation rate (%)**	**Pupal weight (mg)**
0.5% DMSO CK	85±6	43±2
1 µg/g	90±5	44±2
5 µg/g	85±6	45±2
10 µg/g	77±9	45±2
20 µg/g	78±2	44±3
50 µg/g	55±10	40±5
100 µg/g	0	0

Table 2 Toxicity of ochratoxin A to 1st instar larvae of NOW (*A. transitella)*

Treatment of OTA	Survival rate on 12th day (%)	Larval weight on 14th day(mg)	Pupation rate (%)
Plain diet	90±9	38±2	90±7
0.25% DMSO CK	85±5	36±2	75±4
1 µg/g	80±9	31±9	80±7
5 µg/g	85±5	33±3	83±5
10 µg/g	85±9	33±2	75±3
20 µg/g	90±7	23±6	77±2
50 µg/g	80±5	26±6	75±4

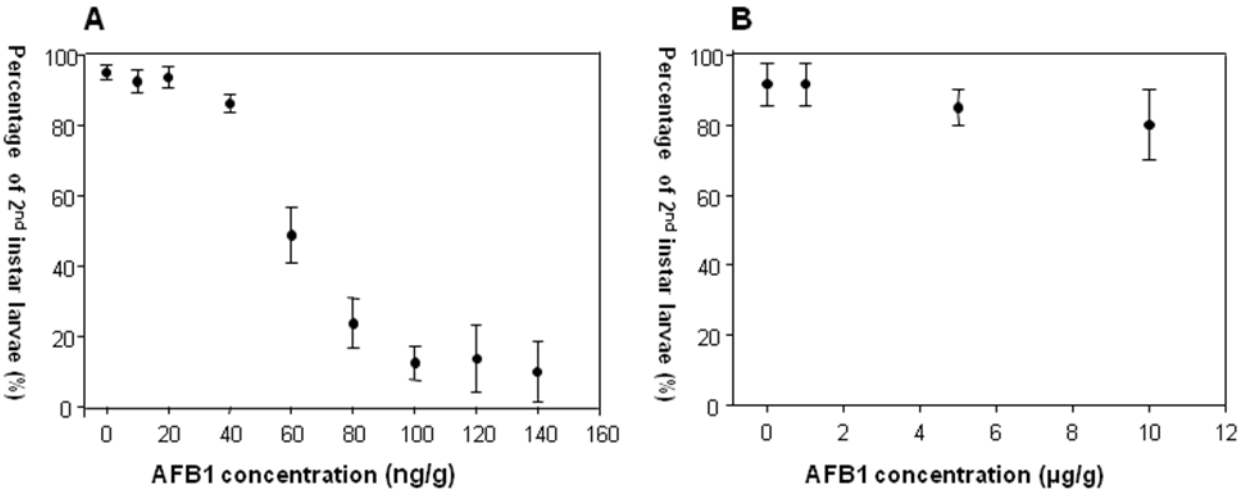

A
Percentage of 2nd instar larvae (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
AFB1 concentration (ng/g)
B
Percentage of 2nd instar larvae (%)
100
80
60
40
20
0
0
2
4
6
8
10
12
AFB1 concentration (µg/g)

Fig. 1

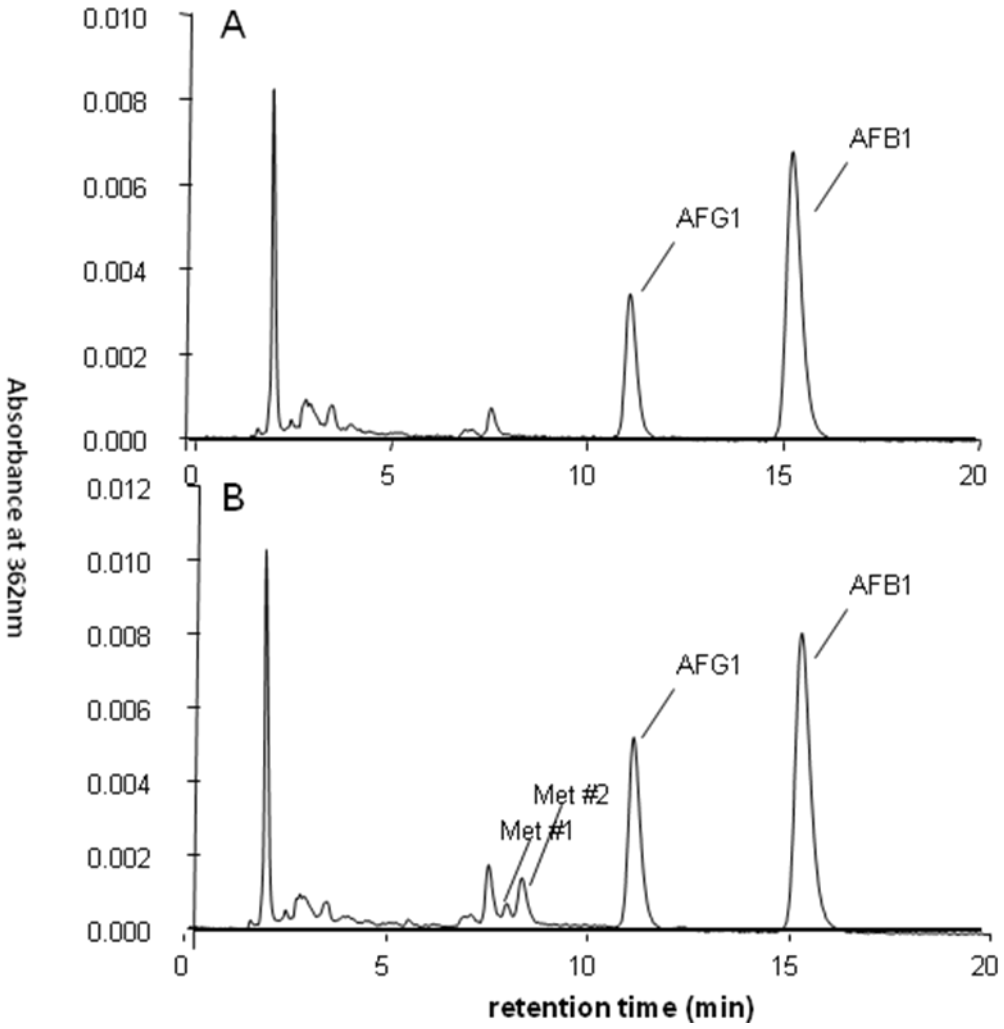
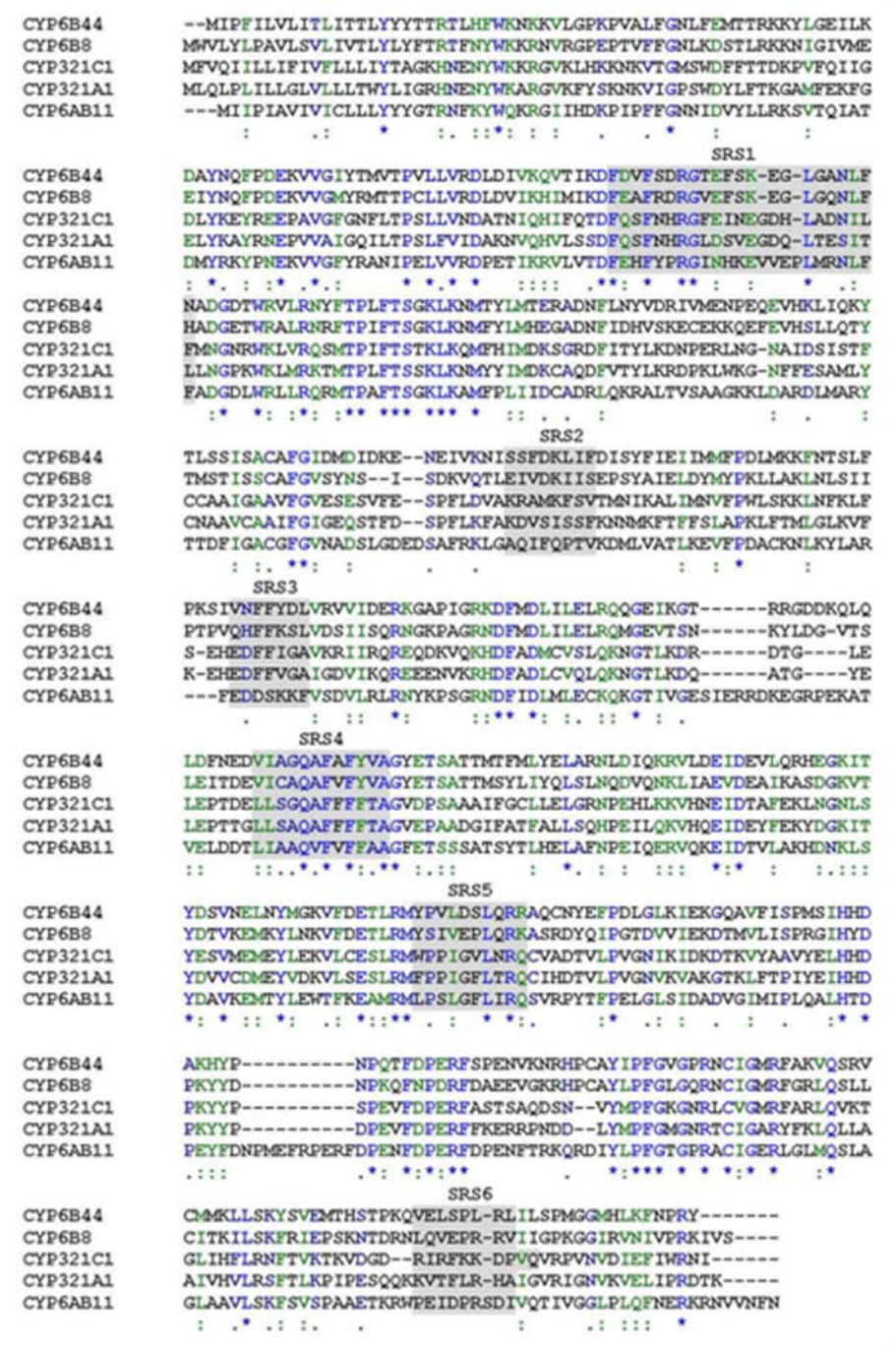

A
B
AFB1
AFG1
Met #2
Met #1
0.010
0.008
0.006
0.004
0.002
0.000
0.012
0
5
10
15
20
Absorbance at 362nm
retention time (min)

Fig. 2


CYP6B44 --MIPFILVLITLITTLYYYTTRTLHFWKNKKVLGPKPVALFGNLFEMTTRKKYLGEILK
CYP6B8 MWVLYLPAVLSVLIVTLYLYFTRTFNYWKKRNVRGPEPTVFFGNLKDSTLRKKNIGIVME
CYP321C1 MFVQIILLIFIVFLLLIYTAGKHNENYWKKRGVKLHKKNKVTGMSWDFFTTDKPVFQIIG
CYP321A1 MLQLPLILLGLVLLLTWYLIGRHNENYWKARGVKFYSKNKVIGPSWDYLFTKGAMFEKFG
CYP6AB11 ---MIIPIAVIVICLLLYYYGTRNFKYWQKRGIIHDKPIPFFGNNIDVYLLRKSVTQIAT
SRS1
CYP6B44 DAYNQFPDEKVVGIYTMVTPVLLVRDLDIVKQVTIKDFDVFSDRGTEFSK-EG-LGANLF
CYP6B8 EIYNQFPDEKVVGMYRMTTPCLLVRDLDVIKHIMIKDFEAFRDRGVEFSK-EG-LGQNLF
CYP321C1 DLYKEYREEPAVGFGNFLTPSLLVNDATNIQHIFQTDFQSFNHRGFEINEGDH-LADNIL
CYP321A1 ELYKAYRNEPVVAIGQILTPSLFVIDAKNVQHVLSSDFQSFNHRGLDSVEGDQ-LTESIT
CYP6AB11 DMYRKYPNEKVVGFYRANIPELVVRDPETIKRVLVTDFEHFYPRGINHKEVVEPLMRNLF
CYP6B44 NADGDTWRVLRNYFTPLFTSGKLKNMTYLMTERADNFLNYVDRIVMENPEQEVHKLIQKY
CYP6B8 HADGETWRALRNRFTPIFTSGKLKNMFYLMHEGADNFIDHVSKECEKKQEFEVHSLLQTY
CYP321C1 FMNGNRMKLVRQSMTPIFTSTKLKQMFHIMDKSGRDFITYLKDNPERLNG-NAIDSISTF
CYP321A1 LLNGPKWKLMRKTMTPLFTSSKLKNMYYIMDKCAQDFVTYLKRDPKLWKG-NFFESAMLY
CYP6AB11 FADGDLWRLLRQRMTPAFTSGKLKAMFPLIIDCADRLQKRALTVSAAGKKLDARDLMARY
SRS2
CYP6B44 TLSSISACAFGIDMDIDKE--NEIVKNISSFDKLIFDISYFIEIIMMFPDLMKKFNTSLF
CYP6B8 TMSTISSCAFGVSYNS--I--SDKVQTLEIVDKIISEPSYAIELDYMYPKLLAKLNLSII
CYP321C1 CCAAIGAAVFGVESESVFE--SPFLDVAKRAMKFSVTMNIKALIMNVFPWLSKKLNFKLF
CYP321A1 CNAAVCAAIFGIGEQSTFD--SPFLKFAKDVSISSFKNNMKFTFFSLAPKLFTMLGLKVF
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SRS3
CYP6B44 PKSIVNFFYDLVRVVIDERKGAPIGRKDFMDLILELRQQGEIKGT------RRGDDKQLQ
CYP6B8 PTPVQHFFKSLVDSIISQRNGKPAGRNDFMDLILELRQMGEVTSN------KYLDG-VTS
CYP321C1 S-EHEDFFIGAVKRIIRQREQDKVQKHDFADMCVSLQKNGTLKDR------DTG----LE
CYP321A1 K-EHEDFFVGAIGDVIKQREEENVKRHDFADLCVQLQKNGTLKDQ------ATG----YE
CYP6AB11 ---FEDDSKKFVSDVLRLRNYKPSGRNDFIDLMLECKQKGTIVGESIERRDKEGRPEKAT
SRS4
CYP6B44 LDFNEDVIAGQAFAFYVAGYETSATTMTFMLYELARNLDIQKRVLDEIDEVLQRHEGKIT
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CYP321A1 LEPTTGLLSAQAFFFFTAGVEPAADGIFATFALLSQHPEILQKVHQEIDEYFEKYDGKIT
CYP6AB11 VELDDTLIAAQVFVFFAAGFETSSSATSYTLHELAFNPEIQERVQKEIDTVLAKHDNKLS
SRS5
CYP6B44 YDSVNELNYMGKVFDETLRMYPVLDSLQRRAQCNYEFPDLGLKIEKGQAVFISPMSIHHD
CYP6B8 YDTVKEMKYLNKVFDETLRMYSIVEPLQRKASRDYQIPGTDVVIEKDTMVLISPRGIHYD
CYP321C1 YESVMEMEYLEKVLCESLRMWPPIGVLNRQCVADTVLPVGNIKIDKDTKVYAAVYELHHD
CYP321A1 YDVVCDMEYVDKVLSESLRMFPPIGFLTRQCIHDTVLPVGNVKVAKGTKLFTPIYEIHHD
CYP6AB11 YDAVKEMTYLEWTFKEAMRMLPSLGFLIRQSVRPYTFPELGLSIDADVGIMIPLQALHTD
SRS6
CYP6B44 AKHYP----------NPQTFDPERFSPENVKNRHPCAYIPFGVGPRNCIGMRFAKVQSRV
CYP6B8 PKYYD----------NPKQFNPDRFDAEEVGKRHPCAYLPFGLGQRNCIGMRFGRLQSLL
CYP321C1 PKYYP----------SPEVFDPERFASTSAQDSN--VYMPFGKGNRLCVGMRFARLQVKT
CYP321A1 PKYYP----------DPEVFDPERFFKERRPNDD--LYMPFGMGNRTCIGARYFKLQLLA
CYP6AB11 PEYFDNPMEFRPERFDPENFDPERFDPENFTRKQRDIYLPFGTGPRACIGERLGLMQSLA
SRS6
CYP6B44 CMMKLLSKYSVEMTHSTPKQVELSPL-RLILSPMGGMHLKFNPRY-------
CYP6B8 CITKILSKFRIEPSKNTDRNLQVEPR-RVIIGPKGGIRVNIVPRKIVS----
CYP321C1 GLIHFLRNFTVKTKVDGD--RIRFKK-DPVQVRPVNVDIEFIWRNI------
CYP321A1 AIVHVLRSFTLKPIPESQQKKVTFLR-HAIGVRIGNVKVELIPRDTK-----
CYP6AB11 GLAAVLSKFSVSPAAETKRWPEIDPRSDIVQTIVGGLPLQFNERKRNVVNFN

Fig. 3