Xenovulene A, a Novel GABA-Benzodiazepine Receptor Binding Compound Produced by *Acremonium strictum*

A. MARTYN AINSWORTH, M. INÊS CHICARELLI-ROBINSON*, BRENT R. COPP†, URSULA FAUTH, PETER J. HYLANDS††, JACQUELINE A. HOLLOWAY, MOHAMMED LATIF, GERARD B. O'BEIRNE†††, NEIL PORTER, DIDIER V. RENNO, MICHELLE RICHARDS and NEIL ROBINSON

Xenova Ltd.,
240 Bath Road, Slough Berkshire SL1 4EF, U.K.

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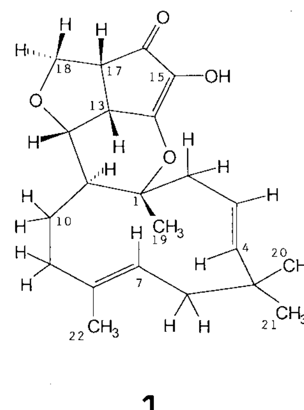
Xenovulene A, a novel inhibitor of benzodiazepine binding to the GABA-benzodiazepine receptor is produced by submerged fermentation of *Acremonium strictum*. It was isolated from the mycelium by solvent extraction and purified by chromatography on Sephadex LH-20 and octadecyl silica. The structure of xenovulene A was determined to be a novel oxygenated sesquiterpene containing a humulene moiety by interpretation of various spectroscopic data, especially from 2D NMR experiments. Xenovulene A inhibited binding of the benzodiazepine, flunitrazepam, with an IC_{50} of 40 nM in an *in vitro* assay using bovine synaptosome membrane preparations.

Benzodiazepine is the conventional term used to describe the structurally related compounds with the basic 1,4-benzodiazepine structure[1]. Pharmacological screening of members of this class of compounds revealed the benzodiazepines to have tranquillising and anticonvulsant activity[2]. Further investigation resulted in the launch of chlordiazepoxide (Librium) for therapeutic use in 1960.

Extensive studies have established that the benzodiazepine mechanism of action involves allosteric modulation of the major inhibitory neuro-transmitter, gamma-aminobutyric acid (GABA). Activation of the $GABA_A$ receptor by GABA agonists causes the Cl^- channel to open. The ensuing influx of Cl^- anions inhibits neuronal firing by causing hyperpolarisation. Benzodiazepines increase the frequency of channel opening without significantly changing the channel conductance or duration of opening. A large number of benzodiazepines have been marketed as tranquillisers. Although effective, prolonged benzodiazepine therapy can lead to the development of dependence in many patients. Side-effects include the development of tolerance, and sedation and acute withdrawal can result in rebound anxiety.

During a screening programme for new inhibitors of the binding of flunitrazepam to the GABA benzodiazepine receptor, we discovered a novel family of active compounds from fermentation of a strain of the fungus *Acremonium strictum*[3]. In the present paper, we describe the taxonomy of the producing strain, fermentation, isolation, structure elucidation and biological activity of the first member of this family, xenovulene A (**1**, Fig. 1). Optimisation of the fermentation conditions and the scale-up for the production of xenovulene A, as well as details of the pharmacological evaluation of xenovulene A and related metabolites will be the subject of further communications.

Fig. 1. Structure of xenovulene A and numbering system.



Present address:
† University of Auckland, Department of Chemistry, Private Bag 92019, Auckland, New Zealand.
†† Phytopharmaceuticals Inc., 887G Industrial Road, San Carlos, California 94070, U.S.A.
††† Amersham International Plc, Cardiff Laboratories, Forest Farm Whitchurch, Cardiff, Wales, CF4 7YT.
This manuscript is a special contribution in honour of Professor SATOSHI ŌMURA's 60th birthday.

Results

Taxonomy of Fungus X11458

This fungus was referred to *Acremonium strictum* W. Gams on the basis of its morphological characteristics following inoculation on 2% malt extract agar. After 10 days the white-pink, dense and compact mycelium reached 15~20 mm diameter at 20°C and had an irregular margin of mainly aerial hyphae. Most conidiophores (up to 40~54 μm long × 1.5 μm wide at the base) were erect (orthotropic), entirely aerial and tended to become bunched (synnematous). They were straight, hyaline, gradually tapered and lacked any chromophilous region. Conidia were cylindrical (3.0~7.0 × 1.5 μm) and copiously produced in slimy masses. Following conidiogenesis on 2% malt extract agar at 24°C, conidia germinated *in situ* resulting either in the production of vegetative hyphae or of conidiophores without an intervening mycelial phase (microcyclic conidiation).

Fermentation

A typical fermentation profile is shown in Fig. 2. The growth of the organism occurred during the first 24 hours and is indicated by the increase in CO_2 emission rate (CER). At the end of the growth phase the dry cell weight reached a concentration of 12 g/liter. The initial pH value of 6.0 remained constant until harvest. During the fermentation a change in morphology of the organism was observed, which was linked to the onset of xenovulene A production. The organism was mainly filamentous during the early exponential phase of growth (Fig. 3a). Conidia were produced as the growth rate was decreasing (Fig. 3b). The production of **1** reached a maximum of 2.9 mg/liter after 4 days of fermentation.

Isolation

Compound **1** was isolated from the mycelium by solvent extraction and purified by chromatography on Sephadex LH-20 and octadecyl silica. Details of the

Fig. 2. Typical fermentation profile of a 10-liter fermentation of *Acremonium strictum*.

■ Xenovulene A produced (mg/liter), (——) CO_2 evolution rate (mmol/liter/hour), (------) Dissolved oxygen tension (%).

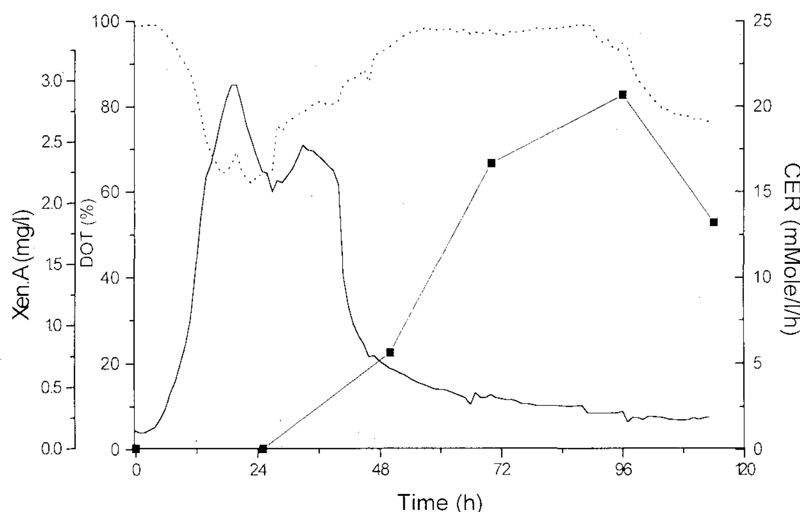
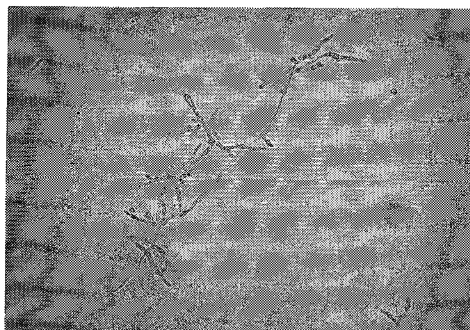


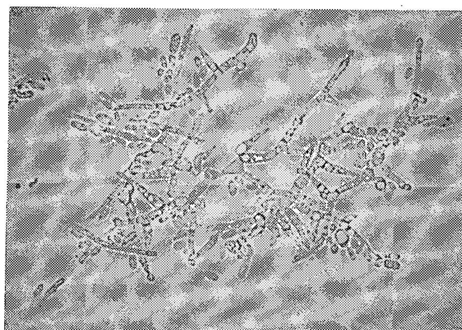
Fig. 3. Morphological change of *Acremonium strictum* during growth in a fermenter.

(a) Vegetative mycelium in the growth phase, (b) conidiating mycelium in the production phase.

(a)



(b)



isolation procedure are described in the experimental section.

Physico-chemical Properties and Structure Elucidation

The physico-chemical properties of **1** are summarised in Table 1. **1** is soluble in methanol, benzene, acetone, ethyl acetate and alkali, but is poorly soluble in water and hexane.

^{13}C and ^{1}H NMR spectra (MeOH-d_4) exhibited 22 carbon and 29 proton signals, respectively, and are summarised in Table 2. As the molecular formula was determined to be $C_{22}H_{30}O_4$ by HR-MS, this indicated the presence of one exchangeable proton. The addition of one acetyl upon reaction of **1** with acetic anhydride-pyridine and the addition of one methyl upon treatment with diazomethane, confirmed the presence of one exchangeable proton and indicated that it was an acidic hydroxyl proton.

The DEPT spectra revealed the presence of four-CH_3, five-CH_2, seven-CH and six quaternary carbons. An H-C correlation experiment established the connectivity of the proton and carbon atoms (see Table 2). Consideration of the chemical shift data indicated that the 70.86 ppm CH_2, the 83.70 ppm CH and the quaternary carbons at 88.94 and 166.96 ppm were attached to oxygen. The presence of four double bonds was deduced from the presence of six olefinic carbons (121.90, 124.68, 135.25, 137.39, 144.73 and 166.96 ppm) and a carbonyl (200.99 ppm), requiring four rings to satisfy the molecular formula.

Extensive connectivity was established through interpretation of ^{1}H-^{1}H 2D COSY and ^{13}C-^{1}H 2D COLOC spectra, leading to the fragment shown in Fig. 4.

The ether linkage between the CH_2 at 70.86 ppm and the methine at 83.70 ppm was deduced from a $^{3}J_{CH}$ coupling between ^{13}C 83.70 ppm and ^{1}H 4.03 ppm, and by the fact that the CH_2 is diastereotopic. The remaining carbon (135.25 ppm) must form a double bond with the 166.96 ppm carbon. Furthermore, the established requirement for an acidic hydroxyl and four rings led to the recognition that the 135.25 ppm carbon forms part of an enol, that the carbonyl is attached to the enol and that the carbons at 88.94 and 166.96 ppm are linked

Table 1. Physico-chemical properties of xenovulene A (**1**).

Appearance	White powder
Molecular weight	358
Molecular formula	$C_{22}H_{30}O_4$
HREI-MS (m/z)	
Calcd:	358.2144 for $C_{22}H_{30}O_4$
Found:	358.2151
HRCI-MS (m/z)	
Calcd:	359.2222 for $C_{22}H_{31}O_4$
Found:	359.2270
UV MeOH λ_{max} nm (ε)	278 (14,000), end absorbance
UV MeOH + 0.1 M KOH λ_{max} nm (ε)	313 (11,500)
$[\alpha]_D$	+520 (c 0.08, MeOH)
IR ν_{max} (KBr) cm^{-1}	3395 (OH) 2930 (CH_2, CH_3) 1730 (C=O)

Table 2. ^{13}C and ^{1}H NMR data of xenovulene A in MeOH-d_4 (30°C).

Position	δC[a]	δH[b]
1	88.94	
2	45.17	2.70 (1H, br ddd, 14.5, 2.2, 1.6), 2.38 (1H, dd, 14.5, 9.9)
3	121.90	5.32 (1H, ddd, 15.9, 10.0, 2.2)
4	144.73	5.24 (1H, dd, 15.9, 1.5)
5	39.37	
6	43.11	2.33 (1H, br dd, 12.7, 12.7), 1.85 (1H, br dd, 13.0, 4.5)
7	124.68	5.17 (1H, m)
8	137.39	
9	37.37	2.23 (1H, br dd, 11.6, 11.6), 2.08 (1H, br dd, 12.5, 7.1)
10	32.24	1.58 (1H, br ddd, 13.2, 10.1, 6.8), 1.37 (1H, m)
11	45.01	2.22 (1H, br dd, 9.8, 5.2)
12	83.70	3.83 (1H, br dd, 6.0, 6.0)
13	42.22	3.67 (1H, dd, 5.8, 5.8)
14	166.96	
15	135.25	
16	200.99	
17	48.97	3.02 (1H, ddd, 7.8, 5.6, 1.5)
18	70.86	4.03 (1H, br dd, 9.3, 1.5), 3.80 (1H, dd, 9.3, 7.9)
19	23.71	1.51 (3H, s)
20	24.92	1.14 (3H, s)
21	30.85	1.13 (3H, s)
22	17.66	1.72 (3H, s)

[a] Chemical shifts are shown referenced to MeOH-d_4 as 49.00 ppm.
[b] Chemical shifts are shown referenced to MeOH-d_4 as 3.40 ppm. The J values are in parenthesis (Hz).

Fig. 4. Structural fragments established through interpretation of ^{1}H-^{1}H 2D COSY and ^{13}C-^{1}H COLOC NMR spectra.

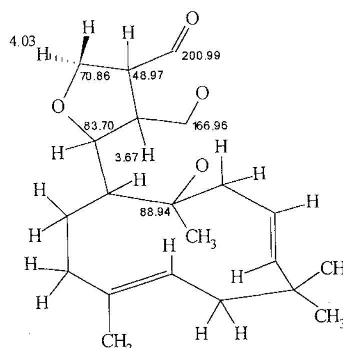
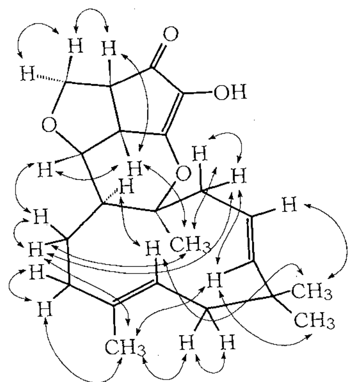


Fig. 5. Observed NOE interactions from a ^{1}H-^{1}H 2D-NOESY experiment.



through a single oxygen. The chemical shift of 200.99 ppm and the carbonyl stretch of 1730 cm^{-1} are both consistent with an $\alpha\beta$-unsaturated ketone in a five-membered ring. Additional evidence was obtained through an ^{1}H-^{13}C 2D HMBC (5 Hz) spectrum in which correlations were observed between the proton at 3.67 ppm and the carbons at 48.97, 135.25, 166.96 and 200.99 ppm.

The relative stereochemistry of **1** was established through ^{1}H-^{1}H 2D NOESY spectra (Fig. 5). Thus, the structure of **1** was elucidated as shown in Fig. 1; subsequently, this was confirmed by X-ray crystallographic studies which will be reported elsewhere.

Benzodiazepine-Receptor Binding Activity

Compound **1** inhibited flunitrazepam binding to the GABA-benzodiazepine receptor with an IC_{50} of 40 nM. The sodium salt has an IC_{50} of 10 nM in the assay.

Discussion

The 11-membered ring in xenovulene A is derived from the sesquiterpene humulene. Humulene occurs in many essential oils, especially of hops, but is also produced in small amounts by a strain of the fungus *Coriolus consors*[4] and is an intermediate in the biosynthesis of many fungal cyclic sesquiterpenoids[5]. Humulene undergoes facile microbial epoxidation of the 6~7 double bond, which in **1** is the point of attachment of the oxygenated tricyclic moiety. Biosynthetically this tricyclic moiety is probably derived *via* a polyketide pathway.

Recently, two very similar fungal metabolites, pycnidione[6] and eupenifeldin[7,8] were reported, which also appear to have a mixed biosynthesis involving the condensation of a sesquiterpene such as humulene. In addition, a higher plant metabolite, lucidene[9], possesses a similar 11-membered ring system. All three compounds contain a humulene ring fused with aromatic moieties at positions 6~7, the most reactive, and 2~3, the second most reactive, double bonds.

In conclusion, through our screening programme we have discovered a new potent inhibitor of flunitrazepam binding to the GABA-benzodiazepine receptor, that is structurally distinct from the benzodiazepines and which possesses a novel carbon skeleton.

Materials and Methods

General Procedures

UV spectra were recorded on a Perkin Elmer Lambda 17 UV/VIS spectrometer. IR spectra were acquired by diffuse reflectance (KBr) on a Nicolet 5PC FT-IR spectrometer. Mass spectra were obtained with a Finnigan Mat 95 spectrometer. A desorption probe was used for both EI and CI spectra and NH_3 was used as CI gas. NMR spectra were recorded on a Bruker ACF400 NMR spectrometer.

Source of Organism

The microfungus designated Xenova culture collection number X11458 was isolated from foam in a tropical forest stream during 1989. It was deposited at the International Mycological Institute, Egham, U.K., on 7 October 1992 under accession number IMI 354451.

Fermentation

A conidial suspension consisting of 4×10^7 conidia of *Acremonium strictum* X11458 was inoculated into a 2-liter Erlenmeyer flask containing 400 ml of seed medium (glycerol, D-glucose, malt extract, soybean peptone, NaCl, $CaCO_3$, Tween 80 (Sigma), Antifoam A (Sigma), adjusted to pH 6.0 with sulphuric acid before sterilisation) and incubated at 25°C, on an orbital shaker at 240 rpm. After 3 days the seed culture was transferred into a 14-liter fermenter containing 10 liters production medium (D-glucose, yeast extract, MES, Tween 80, Antifoam A, carboxymethyl cellulose, adjusted to pH 6.0 with sulphuric acid before sterilisation). The fermentation was carried out at 25°C for 5 days, under 5 liters/minute aeration and 350 rpm agitation. Excessive foaming during the fermentation was controlled by automatic addition of 10% v/v Antifoam A solution.

Isolation

The broth (10 liters) was centrifuged and the mycelium (344 g) extracted with acetone (3×5 liters). The extract was concentrated to an aqueous residue under reduced pressure and then back extracted with EtOAc. The EtOAc extract was evaporated under reduced pressure, redissolved in MeOH and separated by column chromatography on Sephadex LH-20 (3×40 cm; MeOH eluent). Fractions were monitored by analytical reversed phase HPLC for the presence of **1**. Those fractions containing **1** were pooled and purified further by reversed phase preparative HPLC on a C_{18} column (Waters Delta-Pak C_{18}, 100 Å, 15 μm, 25×100 mm) eluting with a mixture of CH_3CN-H_2O (60:40) at a flow rate of 12 ml/minute.

Methylation of **1**

Xenovulene A (6 mg) dissolved in MeOH was treated with an excess of freshly prepared ethereal CH_2N_2. After allowing the reaction to proceed for 10 minutes, the reaction mixture was evaporated to dryness under a stream of nitrogen. The product was purified by preparative reversed phase HPLC (C_{18}, 25 × 100 mm, H_2O-MeCN gradient) to yield 4.7 mg of 15-methoxy **1**: CI-MS *m/z* 373 $(M+H)^+$; EI-MS *m/z* 372 (M, 85%) 233 (80), 215 (80), 203 (100), 169 (20); HREI-MS *m/z* calcd for $C_{23}H_{32}O_4$: 372.2301, found 372.2292; UV λ_{max} (H_2O-MeCN) 268 nm; IR ν_{max} (KBr) cm^{-1} 2944, 2865, 1703, 1632, 1441, 1379, 1337, 1128, 1046; ^1H NMR (400 MHz, MeOH-d_4) δ: 1.13 (6H, s, 20, 21-H), 1.37 (1H, dd, J=13.8, 11.1 Hz, 10_a-H), 1.52 (3H, br d, J=0.3 Hz, 19-H), 1.59 (1H, br ddd, J=14.0, 10.2, 7.2 Hz, 10_b-H), 1.75 (3H, s, 22-H), 1.86 (1H, br dd, J=13.0, 4.6 Hz, 6_a-H), 2.10 (1H, br dd, J=12.2, 7.0 Hz, 9_a-H), 2.23 (1H, br dd, J=11.5, 11.5 Hz, 9_b-H), 2.26 (1H, br dd, J=9.8, 4.9 Hz, 11-H), 2.34 (1H, br dd, J=12.7, 12.7 Hz, 6_b-H), 2.41 (1H, dd, J=14.6, 9.8 Hz, 2_a-H), 2.71 (1H, ddd, J=15.0, 2.2, 1.6 Hz, 2_b-H), 3.02 (1H, ddd, J=8.0, 5.8, 1.6 Hz, 17-H), 3.73 (1H, dd, J=6.0, 6.0 Hz, 13-H), 3.82 (1H, dd, J=9.4, 8.0 Hz, 18_a-H), 3.86 (1H, br dd, J=4.9, 4.9 Hz, 12-H), 3.92 (3H, s, 15-OCH_3), 4.04 (1H, br dd, J=9.4, 1.6 Hz, 18_b-H), 5.17 (1H, m, 7-H), 5.22 (1H, ddd, J=16.0, 9.6, 2.1 Hz, 4-H), 5.29 (1H, dm, J=16.0 Hz, 3-H); ^{13}C NMR (100 MHz, MeOH-d_4) δ: 17.85 (C-22), 23.68 (C-19), 25.11 (C-20), 30.96 (C-21), 32.36 (C-10), 37.55 (C-9), 39.61 (C-5), 42.49 (C-13), 43.27 (C-6), 45.05 (C-11), 45.28 (C-2), 49.16 (C-17), 59.91 (CH_3O), 71.23 (C-18), 83.30 (C-12), 90.20 (C-1), 121.58 (C-3), 124.85 (C-7), 137.68 (C-8), 137.86 (C-15), 145.37 (C-4), 172.26 (C-14), 202.07 (C-16).

Acetylation of **1**

Xenovulene A (5 mg) was dissolved in 2 ml pyridine-acetic anhydride (1 : 1). The solution was stirred at room temperature (18 hours) and dried under vacuum to afford a tan coloured gum. The product was purified by reversed phase HPLC (Waters C_{18} Nova-Pak, 8 × 100 mm, H_2O-MeCN gradient) to yield 15-acetyl **1**: CI-MS *m/z* 401 [$(M+H)^+$, 100%], 359 (20), 205 (15); EI-MS *m/z* 400 (M, 1%), 358 (2), 203 (20), 197 (20), 155 (25); HRCI-MS *m/z* calcd for $C_{24}H_{33}O_5$: 401.2328, found 401.2340; UV λ_{max} MeOH nm (ε) 202.6 (6,200), 257.3 (10,600), UV λ_{max} (MeOH+KOH) nm (ε) 215.1 (3,300), 312.8 (7,400); IR ν_{max} (KBr) cm^{-1} 2946, 1777, 1713, 1642, 1387, 1198, 1117, 1042; ^1H NMR (400 MHz, MeOH-d_4) δ: 1.13 (3H, s), 1.15 (3H, s), 1.37 (1H, br t, J=13.5 Hz), 1.53 (3H, s), 1.59 (1H, m), 1.72 (3H, br s), 1.85 (1H, dd, J=13.0, 4.0 Hz), 2.10 (1H, dd, J=12.9, 6.9 Hz), 2.21 (1H, d, J=11.3 Hz), 2.28 (2H, m), 2.29 (3H, s, $OCCH_3$), 2.39 (1H, dd, J=14.8, 10.4 Hz), 2.68 (1H, ddd, J=14.7, 2.0, 2.0 Hz), 3.15 (1H, ddd, J=7.7, 5.8, 1.6 Hz), 3.87 (2H, m), 3.94 (1H, dd, J=4.9, 4.8 Hz), 4.09 (1H, dd, J=9.3, 2.0 Hz), 5.17 (2H, m), 5.25 (1H, dd, J=15.9, 1.5 Hz).

Preparation of Membranes for Assay

GABA/Benzodiazepine receptors were prepared from ox cerebral cortex[10]. Dura and blood vessels were removed from the cerebral cortex. The tissue was weighed and homogenised mechanically with 10 × ice cold homogenisation buffer (5 mM Tris, 1 mM EDTA, 0.32 M sucrose, pH 7.4). The homogenate was centrifuged at 1,000 × g for 10 minutes at 4°C. The supernatant was decanted and centrifuged further at 48,000 × g for 30 minutes. The supernatant was discarded and the pellet resuspended in ice cold assay buffer (50 mM Tris, pH 7.4). Membrane preparations were aliquoted and stored at −70°C until required.

Binding Assay

Binding to the receptor was measured using standard receptor binding techniques[11] and the radiolabelled high affinity agonist [^3H]-flunitrazepam. The assay buffer used was 50 mM Tris, pH 7.4. The synaptosome membrane preparation (200 μg protein per well) was preincubated in polystyrene microtitre plates with **1** (highest final concentration 0.1 mg/ml) or diazepam (another high affinity agonist, final concentration 1 μM) to determine non-specific binding and equilibrated prior to addition of [^3H]-flunitrazepam (final concentration 1 nM). The assay mixture was incubated for 1 hour at 4°C. "Bound" was separated from "free" by filtration through Wallac 1205-404 (GF/B) filter mats pre-treated with 0.1% w/v polyethylamine for 30 minutes. The filter mats were placed in a Skatron 96-well harvester and washed with ice cold 50 mM Tris buffer for 10 seconds. After drying, the filter mats were counted on a Wallac β-plate reader using MeltiLex solid scintillant (Wallac 1205-422).

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