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BIOSYNTHESIS OF MYCOSPORINES: MYCOSPORINE GLUTAMINOL IN *TRICHOTHECIUM ROSEUM*

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Key Word Index—*Trichothecium roseum;* Deuteromycete; Mycosporines; Biosynthesis, Shikimate pathway; Mycosporine glutaminol.

Abstract—The Deuteromycete *Trichothecium roseum* produces significant amounts of mycosporine glutaminol during its sporulation phase. This production is improved when glucose is substituted by quinic acid in the culture medium. Data from the incorporation of [1-^{14}C]acetic acid, [1-^{14}C]glyceric acid and [U-^{14}C]3-dehydroquinic acid are in very good agreement with the C_6-C_1 unit (cyclohexenone) of fungal mycosporines originating from the first part of the shikimate pathway.

INTRODUCTION

Mycosporines are water-soluble UV-absorbing fungal metabolites, first discovered in sporulating mycelia [1–3] and localized inside reproductive organs (spores) [2, 4, 5], hence their name given by Favre-Bonvin *et al.*, who elucidated their structures [6, 7].

These natural products contain a substituted cyclohexenone (C_6-C_1; **4**) linked with an amino acid (Ser, Glu or Gln) or with their corresponding amino alcohols [6, 7]. Closely related compounds, iminomycosporines, have been isolated from marine Algae [8], Coelenterates [9–12], Molluscs [13] and Fish (eggs) [14, 15].

The physiological activity of fungal mycosporines still remains misunderstood [5]. However, in some cases, their photoprotective role in *Glomerella cingulata* spores [16, 17] and their sporogenic activity in *Nectria galligena* [18, 19] have been demonstrated.

In order to improve our biochemical knowledge of these compounds and to know from which part of central metabolism the C_6-C_1 unit is derived, a biogenetic study has been undertaken by using cultures of a Deuteromycete, *Trichothecium roseum*, remarkable for its high production of mycosporine glutaminol (**1**). In order to choose between the polyketide or shikimate pathways (Scheme 1), we have first followed the biogenesis of **1** produced by *T. roseum* cultures. We have shown that *T. roseum* is able to grow with large concentrations of quinate, that quinate addition improves the mycosporine production and, finally, we have demonstrated that the C_6-C_1 unit derives from the first part of the shikimate pathway.

RESULTS AND DISCUSSION

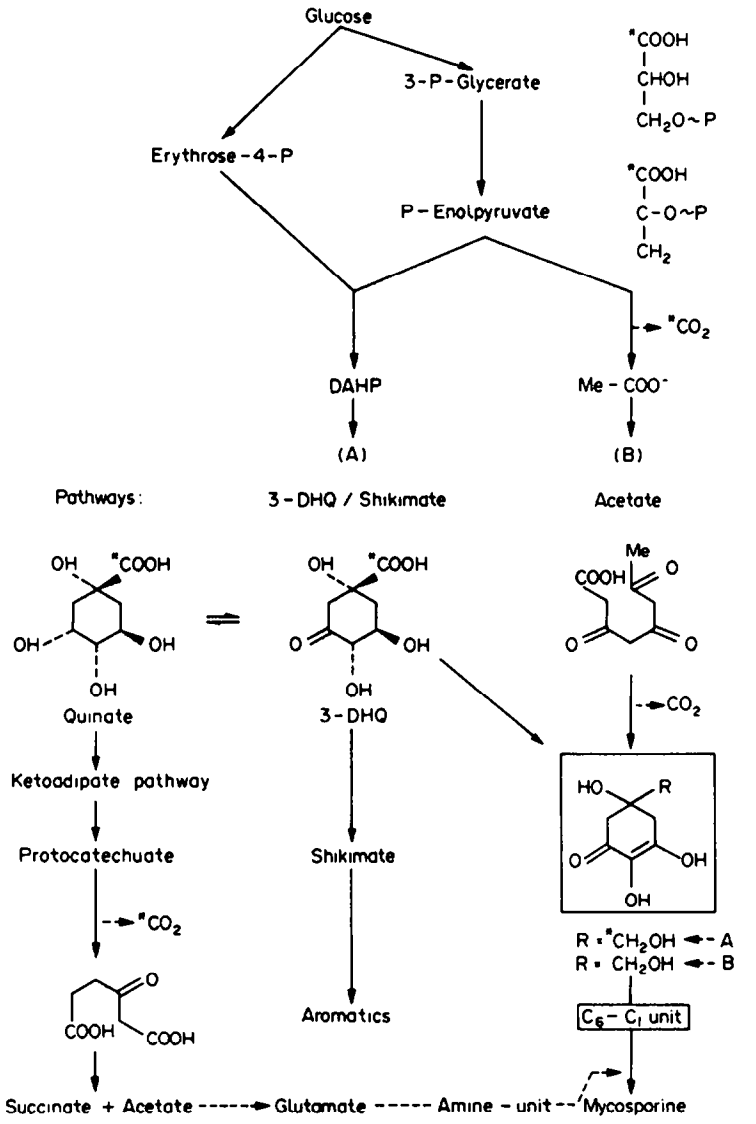
Physiological phases accompanying the development of T. roseum *and mycosporine glutaminol synthesis*

Figure 1 shows the changes observed for several parameters during growth and development of *T. roseum* in batch cultures in GABTO medium (see Experimental). The highest production of biomass occurred between days 8 and 10 corresponding to the depletion of glucose, with a growth yield (g cell/g glucose) of 40%, as usually found [20], and a specific growth rate (μ) equal to 0.043/hr, during the exponential phase.

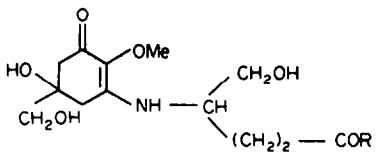
Sporulation and mycosporine synthesis started on day 6 when the sole nitrogen source, asparagine, was depleted. This synchronicity of both events confirms the correlation between both processes [4]. We have observed the pH decreasing during the sporulation process, then rising to seven. The amount of mycosporine in sporulating mycelium reached 2.5% of the dry weight.

Substitution of glucose by quinic acid and asparagine by glutamine in T. roseum *cultures*

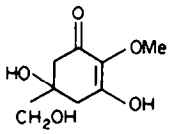
In order to improve the mycosporine production we assayed several compounds in the medium culture, especially quinic acid and glutamine which are potential precursors of **4** and **5**. In the first experiment we progressively substituted glucose by quinic acid in the culture medium which was always adjusted to pH 6.5 before sterilization. As the quinic acid concentration rose, the growth was delayed, but after day 14, the growth yield in



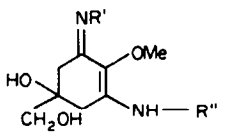
Scheme 1. Possible biosynthetic pathways for formation of the C_6-C_1 unit of mycosporines. DAHP: 3-Deoxy-D-arabinoheptulosonate-7-phosphate, 3-DHQ: 3-dehydroquinate.



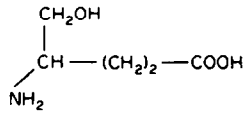
1 R = NH_2 Mycosporine glutaminol
2 R = OH Mycosporine glutamicol



4 Cyclohexenone



3 Iminomycosporine skeleton



5 Glutamicol

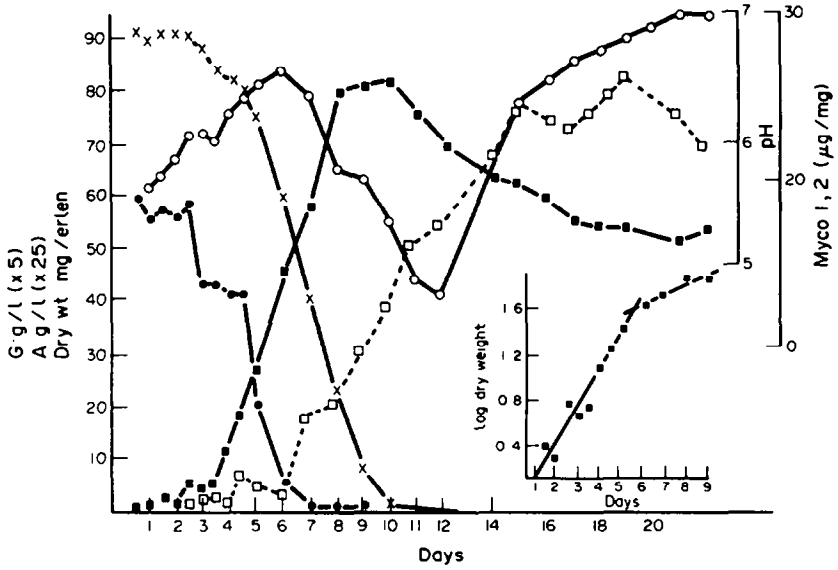


Fig 1. Physiological changes during growth and development of *T. roseum* in batch culture with GABTO medium (see Experimental) at 20° in alternating light–darkness (12–12 hr), 2500 ergs/cm²/s. D.W.■ ■ ■ : dry weight (mg); G: × × × : glucose; A: ● ● ● ● : asparagine; ○ ○ ○ : pH; □ □ □ □ : mycosporines 1, 2.

all cases was at the same level as in the GABTO medium, including when the quinic acid was the sole carbon source. Thus, *T. roseum* synthesizes all the adaptative enzymes required to utilize quinic acid for its growth and development. Moreover, the mycosporine production was clearly increased when quinate was added and the amount was doubled when 70% of carbon was derived from quinate (see Table 1a and Scheme 2). We also noted an increase of pH after the uptake of quinate.

In the second experiment, asparagine was progressively replaced by glutamine, the amino unit of mycosporine glutaminol. Replacement of 80–100% caused a dramatic drop in dry weight and mycosporine production, consecutive to a severe drop of pH (see Table 1b and Scheme 2).

Finally an experiment of double substitution gave results (Table 1c and Scheme 2), showing that the C medium (where quinic acid and glutamine represent *ca* 90% of the carbon and nitrogen sources, respectively (see Experimental) was the most suitable for mycosporine production (× 2.3 in comparison with the A medium: GABTO). Data from the D medium demonstrated that the glutamine can be a good nitrogen source if the pH of the medium is held above six during the developmental process. In all cases the addition of quinic acid improved mycosporine synthesis.

The use of quinic acid as carbon source has already been shown in several fungi, especially in *Neurospora crassa*. In this fungus, in addition to an enzymatic complex leading from DAHP to chorismic acid [21, 21a], an isoenzyme allows the degradation of quinate by the ketoadipate pathway [22]. Our results show that we probably have the same situation with *T. roseum*.

Incorporation of labelled compounds

In order to know the radioactive distribution in **1** after incorporation of labelled compounds, we have to hydrolyse it and purify both components: the cyclohexenone (**4**) and the aminoalcohol (glutaminol in deaminated form, glutamicol, **5**) as already described [23].

Scheme 1 describes both pathways possibly involved in mycosporine biosynthesis. By the ketoadipate pathway, quinic acid also gives acetic acid which is the precursor of the glutamic acid moiety in mycosporines [24]. Thus, the experiment with [U-^{14}C]3-dehydroquinate (3-DHQ) does not appear to be conclusive *per se*. On the other hand, glyceric acid is a direct precursor of 3-DHQ. When labelled at C-1 the label could be found on the C_6-C_1 unit but not on ketoadipic acid and then not on acetic acid as shown in Scheme 1. Therefore, we incorporated [1-^{14}C]acetic acid, [1-^{14}C]glyceric acid and [U-^{14}C]3-DHQ into three cultures of *T. roseum*. After extraction and purification of radioactive **1** to constant specific radioactivity, we determined the specific radioactivity of the purified cyclohexenone (**4**) and glutamicol (**5**).

The data summarized in Table 2 show that: (i) [1-^{14}C]acetic acid was incorporated only into the glutamic moiety (90% of the total radioactivity). (ii) Glyceric acid labelled on the C-1 (not found in the acetate) was only incorporated into the cyclohexenone skeleton (96% of the total radioactivity). (iii) As expected, with [U-^{14}C]3-DHQ, the radioactivity was distributed between the glutaminol moiety (40%) and the C_6-C_1 unit (60%). This indicates that 3-DHQ is a precursor of cyclohexenone as well as of acetate *via* the ketoadipate pathway. Therefore, our experimental data are in good agreement with the hypothesis of the 3-DHQ originating in the first part of the shikimate pathway acting as the precursor for the C_6-C_1 unit of mycosporine. Thus, 3-DHQ appears to be a key compound for anabolic and catabolic pathways as shown in Scheme 2.

The occurrence in fungi of an association between a shikimic pathway product and the glutamine residue is already known, for example anthglutine [25], γ-L-glutaminyl 3,4-dihydroxy benzene and agaritin [26], but, in this last case, there is production of hydrazines by N-N bond formation.

Table 1. Data from partial or total substitution of glucose by quinic acid and asparagine by glutamine

Days	pH 9	pH 14	pH 21	Dry weight (mg) 9	Dry weight (mg) 14	Dry weight (mg) 21	Mycosporines (mg) 9	Mycosporines (mg) 14	Mycosporines (mg) 21
(a)									
GABTO	5.36	6.51	—	70	56.7	—	0.47	0.85	—
	(0.20)*	(0.30)	—	(5.6)	(2.9)	—	(0.08)	(0.19)	—
G_5Q_5ABTO	8.54	9.14	—	84.1	78	—	1.14	1.49	—
	(0.14)	(0.02)	—	(2.5)	(4.119	—	(0.14)	(0.16)	—
G_3Q_7ABTO	8.47	9.47	—	82.3	76.7	—	0.94	1.49	—
	(0.07)	(0.03)		(4.2)	(2.22)		(0.16)	(0.15)	
QABTO	8.60	8.89	—	45.9	72.2	—	0.35	1.19	—
	—	—		—	—		—	—	
(b)									
GABTO	5.72	6.76	—	81.7	64.1	—	0.59	0.87	—
	(0.26)	(0.19)		(3.66)	(5.9)	—	(0.11)	(0.26)	—
GA_8Gl_2BTO	5.77	6.74	—	75.3	62	—	0.65	0.83	—
	(0.19)	(0.06)	—	(3.15)	(1.94)	—	(0.19)	(0.20)	—
GA_6Gl_4BTO	6.05	6.68	—	75.9	64.5	—	0.63	1.23	—
	(0.17)	(0.07)	—	(2.9)	(2.42)	—	(0.08)	(0.11)	—
GA_4Gl_6BTO	5.92	6.95	—	75.5	61.3	—	0.55	0.62	—
	(0.15)	(0.37)	—	(4.9)	(2.92)	—	(0.18)	(0.22)	—
GA_2Gl_8BTO	3.87	4.52	—	44.3	38	—	0.05	0.05	—
	(0.69)	(1.23)	—	(9.96)	(11.1)	—	(0.07)	(0.06)	—
GGlBTO	3.98	3.75	—	18.6	20.5	—	—	—	—
	(0.07)	(0.10)	—	(2.03)	(3.07)	—	—	—	—
(c)									
A medium	5.53	5.78	6.48	73.1	67.6	54.3	0.38	0.95	0.98
	(0.12)	(0.19)	(0.23)	(6.4)	(4.8)	(3.6)	(0.06)	(0.08)	(0.15)
B medium	8.07	9.41	9.68	73.5	83.9	77.3	0.57	0.89	1.23
	(0.26)	(0.06)	(0.14)	(4.8)	(5.5)	(4.2)	(0.11)	(0.08)	(0.09)
C medium	8.56	9.44	9.97	48.9	96.9	78.3	0.47	1.66	2.28
	(0.16)	(0.15)	(0.07)	(8.8)	(5.2)	(6.5)	(0.11)	(0.13)	(0.25)
D medium	8.39	9.07	9.6	17.9	74.3	77.3	0.28	0.95	1.64
	(0.18)	(0.17)	(0.22)	(9.4)	(7.5)	(6.6)	(0.11)	(0.46)	(0.19)

Each value is the mean of 10 replicates.

(a) Partial substitution of glucose by quinic acid.

(b) Partial or total substitution of asparagine by glutamine.

(c) Partial or total double substitution of glucose and asparagin by quinic acid and glutamine respectively.

*Values in parentheses are the standard deviation.

The most outstanding fact which remains to be resolved is the reduction of the α-COOH of the glutamine, which is a very uncommon reaction. However, reduction of alanine to alaninol in bacteria [27], and of *N*-benzoyl phenylalanine to *N*-benzoyl phenylalaninol in *Penicillium* sp. are reported [28].

EXPERIMENTAL

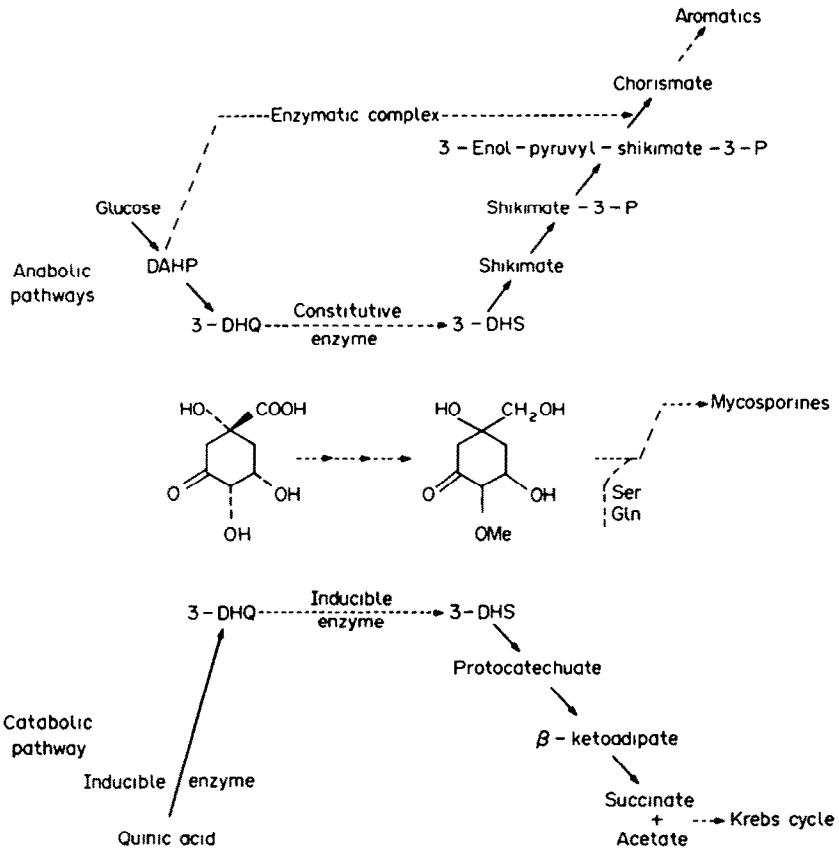
Growth and development of Trichothecium roseum. The Deuteromycete *Trichothecium roseum* (Pers.) Link ex Gray, strain 281-28 was purchased from Centraal Bureau voor Schimmelcultures, Baarn, Holland. The basic medium culture, A medium (GABTO: Glucose, Asparagine, Biotin, Thiamine and Oligoelements) contains (g/l): glucose (20), asparagine (2.6), KH_2PO_4 (1.75), $MgSO_4 \cdot 7H_2O$ (0.75), biotin and thiamine (25×10^{-6} each); trace elements: Zn, Fe, Cu, Mn, B, Mo. After adjustment to pH 6.5, this medium was dispersed into 25 ml conical flasks (300) each containing 10 ml. After sterilization (25 min at 120°) each flask was inoculated with 20 μl of spore suspension (1600 spores/μl). The flasks are placed in a thermostated room at 20 ± 1°, in alternating light and darkness (12–12 hr; 2500 ergs/cm²/sec).

At regular intervals (12 then 24 hr), the content (mycelium and culture medium) of 12 flasks was analysed. The remaining substrate was analysed by the GOD-PAP enzymatic method (Boehringer) for glucose and by HPLC, after dansyl derivatization, for asparagine [23].

The mycelium was recovered by filtration, washed, lyophilized, weighed and extracted by boiling EtOH–H_2O (1:1), and the amount of mycosporine glutaminol (1) determined by UV detection ($E^{1\%}_{1cm}$: 890) [23].

Substitution of glucose and asparagine by quinic acid and glutamine respectively. In partial or total substitution experiments of glucose and asparagine we used the media shown in Table 3 without changes in the minerals and vitamins contents of A medium.

Incorporation, purification and dosage of labelled compounds. The labelled precursors were added into the medium culture 4 days after inoculation (see Table 1); 15 days after inoculation the mycelium was filtered, washed × 3 with H_2O, and



Scheme 2. Anabolic (aromatic and mycosporines compounds) and catabolic (succinate, acetate derivatives) pathways from the 3-dehydroquinic acid postulated in *T. roseum*, (adapted from [22]). DAHP: 3-deoxy-D-arabinoheptulosonate-7-phosphate, 3-DHQ: 3-dehydroquinate, 3-DHS: 3-dehydroshikimate.

Table 2. Distribution of radioactivity into mycosporine glutaminol (1) after addition of labelled [^{14}C] precursors

Precursors	[1-^{14}C]Acetic acid	[1-^{14}C]Glyceric acid	[U-^{14}C]3-Dehydroquinic acid
KBq added in the culture medium	9250	925	555
Specific activity MBq/mM	2035	1961	
Incubation time (days after inoculation)	4	4	4
KBq recovered in the medium	1000 (11%)	81,4 (9%)	35,2 (6%)
Amount of mycosporine glutaminol (1) recovered (mg)	3,1	3,5	1,14
Specific activity of 1, KBq/mM	1280	141	47,4
Specific activity of cyclohexenone 4, KBq/M	118	162	18,9
Specific activity of glutamicol 5 KBq/mM	1254	5,90	26,5
Distribution of the radioactivity in 1, ratio 4/5	10/90	96/4	40/60

extracted twice by a boiling mixture of 10 ml of EtOH–H_2O (1:1). After evaporation, the residue was washed with EtOAc (3 × 2 ml) for solubilization of lipid constituents, then dissolved in 1 ml H_2O. The muddy soln was filtered on cotton and put at the top of a column (10 × 1 cm) of Dowex 50 AG 2 × 8, 50–100 mesh, OH^- form, and eluted with H_2O. Mycosporine glutaminol (1) was recovered in the first 10 ml, to which was added Dowex 50 W × 8, 100–200 mesh. After stirring for 10 min, 1 was almost entirely adsorbed on the resin, as shown by the UV spectrum of the supernatant. The resin was filtered and washed with H_2O (3 × 1 ml) in order to remove sugars and non-cationic compounds. Compound 1 was allowed to stay in the adsorbed form on the cationic resin for 4 hr at 40° to deaminate 1 to 2, as shown in ref. [23]. After elution with conc NH_4OH, 2 was evapd to dryness at red. pres. and the residue dissolved in aq. 0.05% AcOH, before two successive purifications by HPLC on (a) RP-18 column 7 μm

Table 3. Growth media used to culture *Trichothecium roseum*

Media	Concentration of substrates (g/l) Glucose (G)	Quinic acid (Q)	Asn (A)	Gln (Gl)
GABTO (A Medium)	20	—	2.6	—
G_5Q_5ABTO	10	10	2.6	—
G_3Q_7ABTO	6	14	2.6	—
QABTO	—	20	2.6	—
GA_8Gl_2BTO	20	—	2.08	0.52
GA_6Gl_4BTO	20	—	1.56	1.04
GA_4Gl_6BTO	20	—	1.04	1.56
GA_2Gl_8BTO	20	—	0.5	2.08
GGlBTO	20	—	—	2.56
B Medium	10	10	1.56	1.04
C Medium	2	18	0.26	2.34
D Medium	—	20	—	2.56

(25 × 1 cm), solvent: 0.05% aq. AcOH at 2 ml/min; UV, 313 nm; R_t: *ca* 32 min; (b) NH_2 column 10 μm (30 × 1 cm); solvent: 0.05% aq. AcOH at 2 ml/min; UV, 313 nm; R_t: *ca* 22 min.

As demonstrated in preliminary experiments with [U-^{14}C]glucose, the above purification give mycosporine glutamicol (**2**) which was radiochemically pure.

Hydrolysis of mycosporine glutamicol (**2**). About 1 mg of **2** was placed in 1 ml of 0.014N HCl, at 100° for 2 hr, then the glutamicol (**5**) was adsorbed on Dowex 50 W × 8, 100–200 mesh, directly added in hydrolysis soln. The cyclohexenone (**4**) remaining into aq. soln was purified by HPLC: NH_2 column 10 μm (30 × 1 cm); solvent: 0.05% aq. AcOH at 2 ml/min; UV, 254 nm; R_t: *ca* 16 min.

After desorption by *ca* 2 ml of conc NH_4OH and concn to dryness, the residue was dissolved into 0.1 ml carbonate buffer (0.2 M, pH 9.7) and 0.1 ml dansyl chloride (0.1% in Me_2CO). After 35 min at 40°, dansyl glutamicol was purified by HPLC; RP-18 column 7 μm (25 cm × 1 cm); solvent: H_2O–MeOH (7:13 with 2% AcOH), 2 ml/min, UV, 254 nm; R_t *ca* 30 min.

In preliminary non radioactive experiments, the purity of the cyclohexenone and dansyl glutamicol were controlled by hydrolysis of **2** (10 mg) followed by ^{1}H NMR and MS measurements of the purified degradation products [23]. ε of **4** (in 1% AcOH) at 268 nm: 15700. ε of dansyl glutamicol (in MeOH) at 250 nm: 19300; at 334 nm: 6430.

Radioactive measurements. Measurements of radioactive compounds were carried out by liquid scintillation counting in the following mixture: Naphthalene (100 g), PPO (7 g), dimethyl POPOP (0.3 g) and dioxan to make 1 l. To 5 ml of this mixture was added 0.5 ml of aq. or alcoholic solns of the test sample in a 10 ml plastic vial. Counting efficiencies were found to be as follows: 5 ml scintillation liquid + 0.5 ml MeOH: 64.8%; 5 ml scintillation liquid + 0.5 ml H_2O: 60.5%; 5 ml scintillation liquid + 0.5 ml 1% aq. AcOH: 57.7%.

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