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Appendix

Table 10 Structures of naturally occuring terahydroisoquinoline monoterpene alkaloids (Structurally related glucosides are also included) in *Alangium salviifolium* Wang

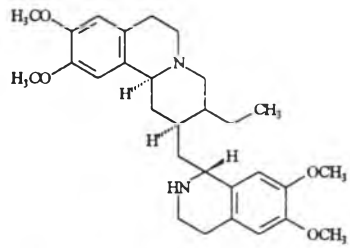
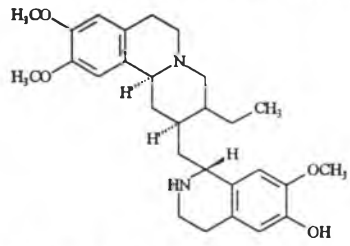
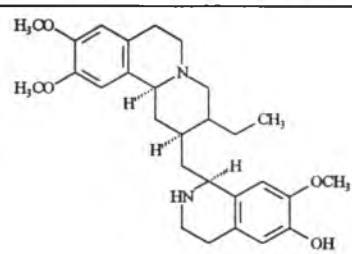
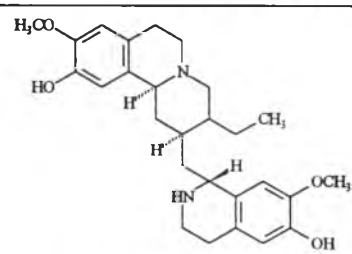
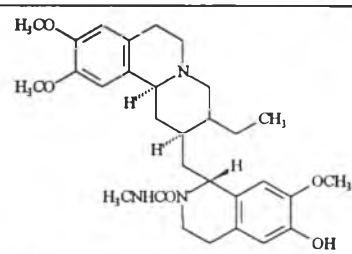
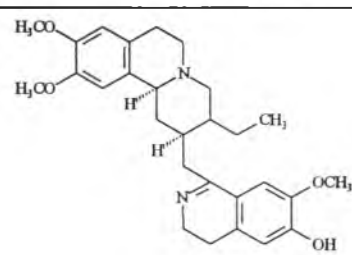
Compound names	Structures	References
1. Emetine		Budzikiewicz, and Pakrashi and Vorburggen,1964
2. Cephaeline		Budzikiewicz, and Pakrashi and Vorburggen,1964
3. Isocephaeline		Achari *et al.*,1980
4. Demethylcephaeline		Pakraki and Achari, 1970
5. Alangamide		Pakraki and Ali, 1969
6. Psychotrine		Budzikiewicz, and Pakrashi and Vorburggen,1964

Table 10 (continues)

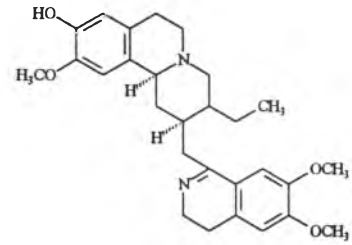
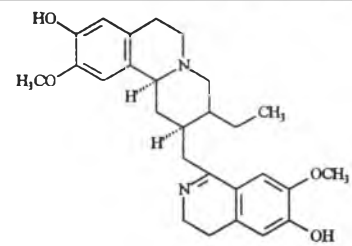
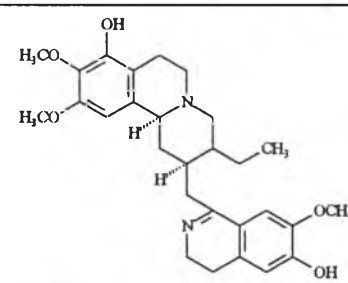
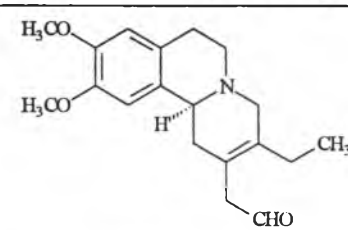
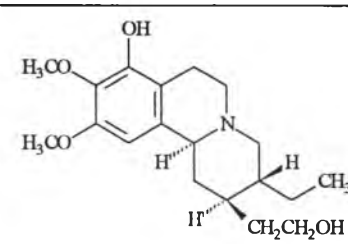
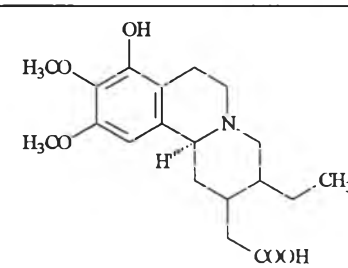
Compound names	Structures	References
7. Demethylpsychotrine		Pakraki and Ali, 1967
8. 11-Hydroxypsychotrine		Willamam and Li, 1970
9. Alangicine		Pakraki and Ali, 1967
10. Dehydroprotoemetine		Willaman and Li, 1970
11. Ankorine		Battersby *et al.*, 1966
12. Alacine		Chattopadhay *et al.*,1984

Table 10 (continues)

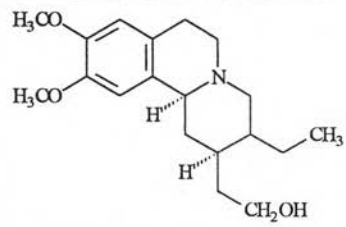
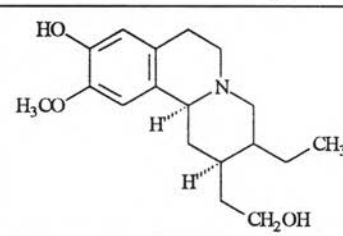
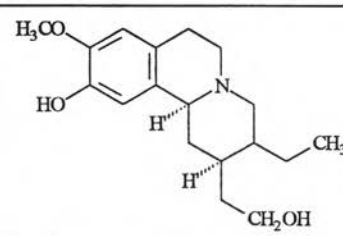
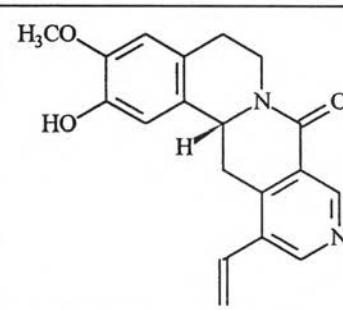
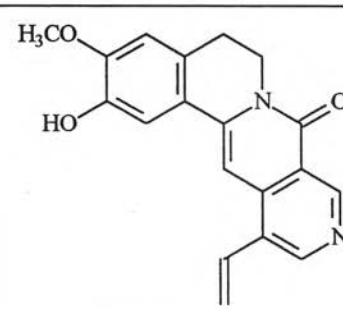
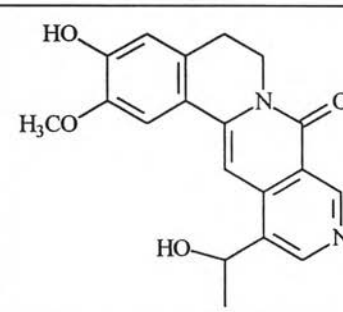
Compound names	Structures	References
13 Protoemetinol		Albright, Van Meter and Goldman, 1965
14. 9-Demethylprotoemetinol		Ali *et al.*, 1982
15. 10-Demethylprotoemetinol		Ali *et al.*, 1982
16. Alangimaridine		Pakrashi *et al.*, 1980
17. Alangimarine		Pakrashi *et al.*, 1980
18. Isoalangimarine		Pakrashi *et al.*, 1985

Table 10 (continues)

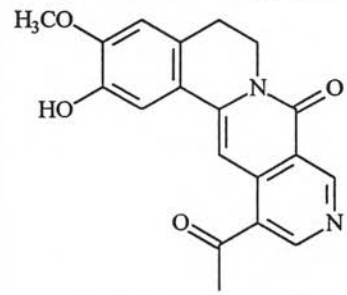
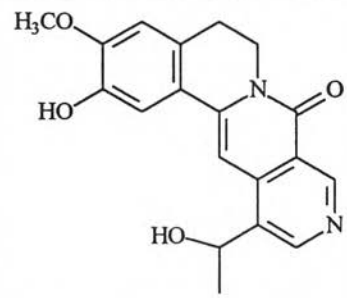
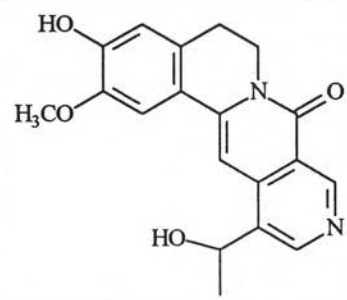
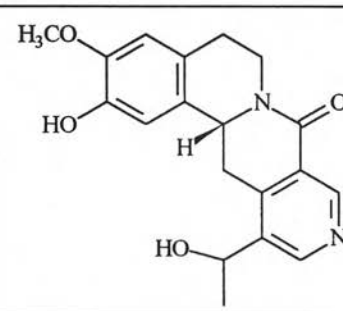
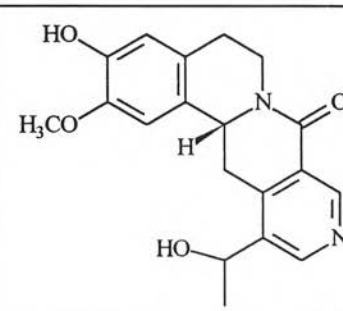
Compound names	Structures	References
19. Alangimarinone		Pakrashi *et al.*, 1980
20. Alamarine		Pakrashi *et al.*, 1980
21. Isoalamarine		Pakrashi *et al.*, 1985
22. Dehydroalamarine		Pakrashi *et al.*, 1985
23. Dehydroisoalamarine		Pakrashi *et al.*, 1985

Table 10 (continues)

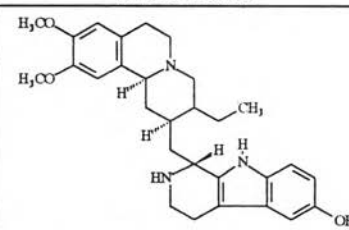
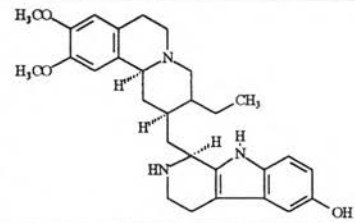
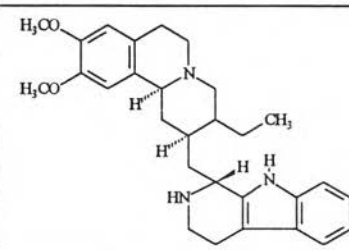
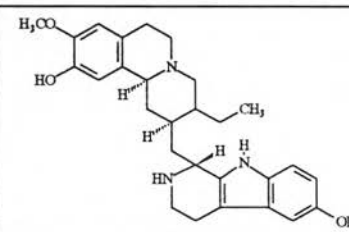
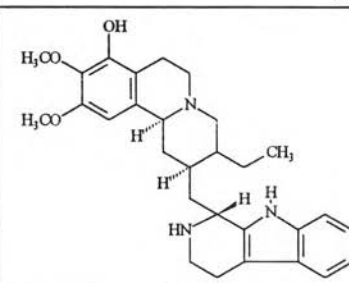
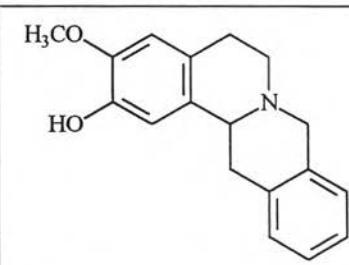
Compound names	Structures	References
24. Tubulosine		Albright, Van Meter and Goldman, 1965
25. Isotubulosine		Popelack, Haack and Spingler, 1966b
26. Deoxtubulosine		Battersby *et al.*, 1965
27. 10-Demethyltubulosine		Popelack, Haack and Spingler, 1966a
28. Alangimarckine		Pakrashi, 1964
29. Bharatamine		Pakrashi, *et al.* 1983

Table 10 (continues)

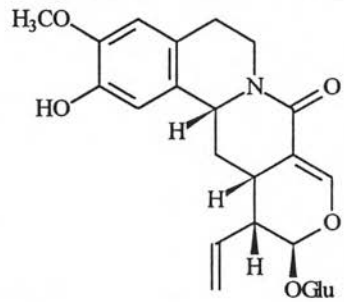
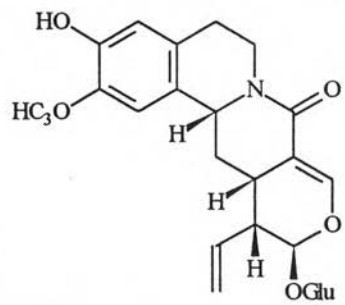
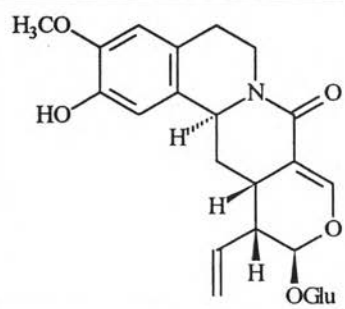
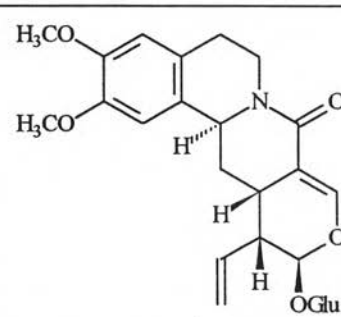
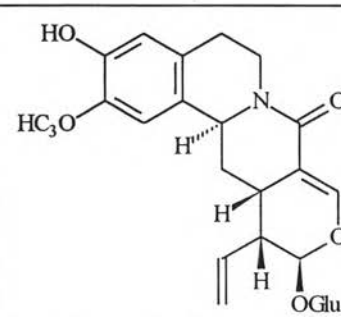
Compound names	Structures	References
30. alangiside		Shoeb *et al.*, 1975
31. 3-*O*-Demethyl 2-*O*-methylalangiside		Itoh *et al.*, 1994
32. Isoalangiside		Itoh, Tanahashi, and Nagakura, 1995
33. Methylisoalangiside		Itoh, Tanahashi, and Nagakura, 1995
34. 3-*O*-Demethyl 2-*O*-methylisoalangiside		Itoh, Tanahashi, and Nagakura, 1995

Table 10 (continues)

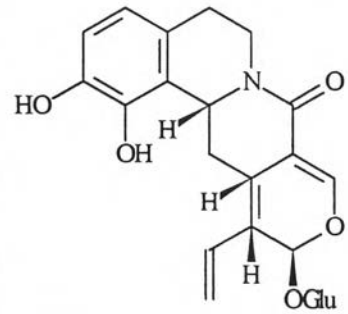
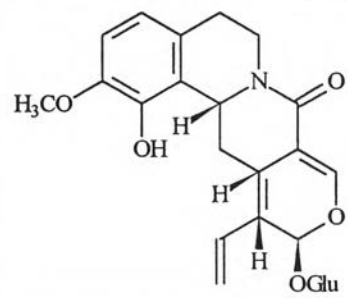
Compound names	Structures	References
35. Demethylneoalangiside		Itoh, Tanahashi, and Nagakura, 1995
36. Neoalangiside		Itoh, Tanahashi, and Nagakura, 1995

Table 11 Ammonium sulfate precipitation table (Harris and Angal, 1989)

The amount of solid of ammonium sulfate to be added to solution to give the desired final saturation at 0°C

Initial concentration of ammonium sulfate	Final concentration of ammonium sulfate, % saturation at 0°C																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	g solid ammonium sulfate to add to 100ml of solution																
0	10.7	13.6	16.6	19.7	22.9	26.2	29.5	33.1	36.6	40.4	44.2	48.3	52.3	56.7	61.1	65.9	70.7
5	8.0	10.9	13.9	16.8	20.0	23.2	26.6	30.0	33.6	37.3	41.1	45.0	49.1	53.3	57.8	62.4	67.1
10	5.4	8.2	11.1	14.1	17.1	20.3	23.6	27.0	30.5	34.2	34.9	41.8	45.8	50.0	54.5	58.9	63.6
15	2.6	5.5	8.3	11.3	14.3	17.4	20.7	24.0	27.5	31.0	34.8	38.6	42.6	46.6	51.0	55.5	60.0
20	0	2.7	5.6	8.4	11.5	14.5	17.7	21.0	24.4	28.0	31.6	35.4	39.2	43.3	47.6	51.9	56.5
25		0	2.7	5.7	8.5	11.7	14.8	18.2	21.4	24.8	28.4	32.1	36.0	40.1	44.2	48.5	52.9
30			0	2.8	5.7	8.7	11.9	15.0	18.4	21.7	25.3	28.9	32.8	36.7	40.8	45.1	49.5
35				0	2.8	5.8	8.8	12.0	15.3	18.7	22.1	25.8	29.5	33.4	37.4	41.6	45.9
40					0	2.9	5.9	9.0	12.2	15.5	19.0	22.5	26.2	30.0	34.0	38.1	42.4
45						0	2.9	6.0	9.1	12.5	15.8	19.3	22.9	26.7	30.6	34.7	38.8
50							0	3.0	6.1	9.3	12.7	16.1	19.7	23.3	27.2	31.2	35.3
55								0	3.0	6.2	9.4	12.9	16.3	20.0	23.8	27.7	31.7
60									0	3.1	6.3	9.6	13.1	16.6	20.4	24.2	28.3
65										0	3.1	6.4	9.8	13.4	17.0	20.8	24.7
70											0	3.2	6.6	10.0	13.6	17.3	21.2
75												0	3.2	6.7	10.2	13.9	17.6
80													0	3.3	6.8	10.4	14.1
85														0	3.4	6.9	10.6
90															0	3.4	7.1
95																0	3.5
100																	0

Table 12 Solution for SDS-polyacrylamide gel electrophoresis (Leammli, 1970)

Solution	Composition	Procedures
Sample buffer	Distilled water 4.0 ml 0.5M Tris-HCl pH 6.8 1.0 ml Glycerol 0.80 ml 10% w/v SDS 1.60 ml β-mercaptoethanol 0.40 ml 0.05% w/v Bromophenol blue 0.2 ml	Dilute the sample at least 1:4 with sample buffer, and heat at 95°C for 4 min.
Running buffer	Tris base 9 g Glycine 43.2 g SDS 3 g to 600 ml with H_2O	Dilute 60 ml 5x stock with 240 ml H_2O for one electrophoresis run. Store at 4°C. Warm to 37°C before use if precipitation occurs.
Lower gel buffer	1.5 M Tris-HCl, pH 8.8: 27.23 g Tris base	Adjust to pH 8.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.
Upper gel buffer	0.5 M Tris-HCl, pH 6.8: 6 g Tris base	Adjust to pH 6.8 with 1N HCl. Make to 100 ml with distilled water and store at 4°C.
Acrylamide stock	Acrylamide/Bis (30%T, 2.67%C): 87.6 g acrylamide 2.4 g N′,N′-bismethyleneacrylamide	Make to 300ml with distilled water. Filter and store at 4°C in the dark (30 days maximum). Acrylamide is a neurotoxin; do not breathe dust or allow to touch skin. Do not mouth pipette.
10% Ammonium persulfate	100 mg ammonium persulfate (APS)	To make the 10% ammonium persulfate solution, dissolve 100 mg APS in 1 ml H_2O. Freshly prepared daily, store at 4°C.
N,N,N′,N′-Tetramethylenediamine		Store at 4°C.
10% SDS	10 g SDS to 100 ml with distilled water	Dissolve 10 g SDS in water with gentle stirring and bring to 100 ml with H_2O.

Table 13 SDS-polyacrylamide gel electrophoresis (linear slab gel)

Step of procedures	Procedures
1. Preparing the gel	Assemble gel sandwich according to the manufacturer's instructions in the case of commercial apparatus (eg. Bio-Rad Mini-Gel). Prepare the separating gel monomer solution and pour the solution smoothly using an automatic pipet. Immediately overlay the monomer solution with water. Allow the gel to polymerize for 45 min. to 1 hr, rinse off the overlay solution. Prepare stacking gel monomer solution. Carefully insert comb into gel sandwich until bottom of teeth reach top of front plate. Pipette the stacking gel solution onto separating gel until solution reaches top of front plate. Allow the gel to polymerize for 30-45 min. After stacking gel has polumerized, remove comb carefully. Place gel into electrophoresis chamber. Add electrophoresis buffer to inner and outer reservoir, making sure that both top and bottom of gel immersed in buffer.
2. Preparing and loading sample	Combine protein sample and sample buffer in an Eppendorf tube. Heat at 100°C for 2-10 min. Spin down protein solution for 1 sec. Introduce sample solution into well using Elec ™ Tip.
3. Running a gel	Attach electrode plugs to proper electrodes. Current should flow towards the anode. Turn on power supply to 200V. When dye front migrate to the bottom of the gel in 40 min., turn off the power supply. Remove electrode plugs from electrodes. Remove the gel plates from electrode assembly. Carefully remove a spacer, gently pry apart the gel plates. Later, the gels are to be stained.

Staining SDS-PAGE Separated Proteins with Coomassie Brilliant Blue and Silver

1. <u>Standard Coomassie Blue Staining and Rapid Coomassie Blue Staining.</u>

(Detection limit : 0.3 to 1.0 μg protein)

Coomassie blue staining is based on non-specific binding of Coomassie blue dye to proteins. Separated proteins are simultaneously fixed and stained in the gel, and then destained to remove the background prior to drying and photographing. The proteins are detected as blue bands on a clear background (Wilson, 1983.)

Stock Solutions

Always wear gloves and use distilled or deionized water.

Standard Staining

Staining Solution (0.025% Coomassie Brilliant blue R 250, 40% methanol, 7% acetic acid)

0.5 g Coomassie Brilliant blue R
800 ml methanol
Stir until dissolved. Then add :
140 ml acetic acid
ddH_2O to 2 L
Filtering is not needed
Store at room temperature for up to 6 months.

Destaining Solution I (40% methanol, 7% acetic acid)

400 ml methanol
70 ml acetic acid
ddH_2O to 1 L
Store at room temperature indefinitely.

Destaining Solution I (7% acetic acid, 5% methanol)

700 ml acetic acid
500 ml methanol
ddH_2O to 10 L
Store at room temperature indefinitely.

Rapid Staining

Rapid Stain Fixing Solution (25% isopropanol, 10% acetic acid)

250 ml isopropanol
100 ml acetic acid
Bring to 1 L with deionized water.

Rapid Coomassie Blue Stain (0.06% Coomassie blue G-250, 10% acetic acid)

0.6 gm Coomassie Blue G-250
100 ml acetic acid
Deionized water to 1 L

Standard Coomassie Blue Protocol

Perform staining at room temperature. Covered plastic trays work well and minimize exposure to methanol and acetic acid vapors. When covers are not used, these procedures should be done in a fume hood. For accelerated staining and destaining, heat the solutions to 45oC. This will reduce the time by 50%.

1. Place the gel in Staining Solution. Use just enough stain so that the gel floats free in the tray. Shake slowly for approximately 4 hours to ovenight.

2. Replace the staining solution with Destaining Solution I. Shake slowly 30 minutes. This removes the bulk of the excess stain.

3. Remove Destaining Solution I and replace with Destaining Solution II. Typically, the Destaining Solution II is changed twice a day until the gel background is clear. Alternatively, addition of Kimwipe tissue to one corner of the staining tray will help remove Coomassie blue from the gel without changing the destaining solution, minimizing the waste volume generated. Replace the tissues when they are saturated with Coomassie blue. Use caution, however, because excessive destaining will lead to loss of band intensity.

4. Store the gel in Destaining Solution II. To minimize cracking, add 1% glycerol to the last destain before drying the gel.

Rapid Coomassie Blue Protocol

1. Place the gel in a container with Rapid Stain Fixing Solution. Shake slowly for 10 to 15 minutes for a 0.75-1.0-mm gel and 30 to 60 minutes for a 1.5-mm thick gel.

2. Replace the fixing solution with Rapid Coomassie Stain. Shake slowly 2 hours to overnight until the bands are visible.

3. Replace with Destaining Solution II until the background is clear. Add Kimwipes as described in step 3 above. Store in 7% acetic acid or ddH_2O.

2. Silver Staining

Detection limit : 2 to 5 ng protein

Silver staining is based on binding of silver ions to sulfhydryl and carboxyl groups of the separated proteins. After electrophoresis, the proteins are fixed, exposed to silver nitrate, and developed to form a black precipitate of silver. The degree of development of the protein bands can be controlled with the amount of time the gel is exposed to the developer (Merril *et al.*, 1984; Morrissey, 1981, Oakley *et al.*, 1980; Switzer *et al.*, 1971). The procedure below is a modification of Morrissey (1981) and uses DTT reduction to improve reproducibility. Development also occurs more slowly than many silver staining protocols, giving more control over the final image.

Stock Solution

Wear gloves and use only glass-distilled water. Glass staining trays are particularly useful because they are easy to clean. Every step can be done at room temperature.

Silver Staining

Cross-linking Solution (10% glutaraldehyde)

20 ml of 50% glutaraldehyde stock
Distilled water to 100 ml.

DTT (dithiothreitol) Solution (5 μg/ml)

5 mg DTT
Bring to 1 L with ddH_2O.

Silver Nitrate Solution (0.1% w/v silver nitrate)

1 g silver nitrate
Distilled water 1 to L.

3% Sodium Carbonate (3% w/v)

60 g sodium carbonate
Bring to 2 L with distilled water, store in glass container.

Developing Solution (3% sodium carbonate, 0.019% formaldehyde)

200 ml of 3% sodium carbonate
100 µl of 37% formaldehyde
Prepare just before use.

Stop Solution (2.3 M sodium citrate)

67.64 g sodium citrate, dihydrate (FW 294.1)
Bring to a final volume of 100 ml with deionized water.

Silver Stain Protocol

1. Place the gel in Destain I (100 ml), 30 minutes to overnight with gentle shaking.

2. Replace with 100 ml of Destain II. Shake slowly for 30 minutes.

3. Discard Destain II and replace with 100 ml cross-linking solution. Shake for 30 minutes.

   Glutaraldehyde is toxic and must be handled in a fume hood.

   For small peptides, incubate with glutaraldehyde overnight to insure retention of the peptides in the gel.

4. Pour off the glutaraldehyde solution and wash gel with several changes of water over 2 hours. Alternatively, the gel can be removed from the glutaraldehyde and placed into 2 liters of water for overnight storage. The next morning, wash 30 minutes in fresh water. Failure to remove completely glutaraldehyde will result in higher background staining.

After the final wash, add DTT Solution and incubate with slow shaking for 30 minutes.

Remove DTT Solution. Drain well, but do not rinse the gel. Add 100 ml of Silver Nitrate Solution. Shake slowly for 30 minutes.

Place the staining tray under running deionized water, swirl for a few seconds, and then dump the rinse water.

Add 50 ml of Developing Solution, swirl briefly, and then discard the solution. Repeat once for a total of two rinses.

This reacts with the excess silver and prevents nonspecific staining of the gel.

Add 100 ml of Developing Solution and shake slowly. Staining occurs slowly at first but then rapidly progresses.

The development process generally takes 5 to 10 minutes.

When the bands look slightly lighter than the desired staining level, remove developer, rinse quickly with water, and add Destain II as the stop solution. Alternatively, 5 ml of citric acid can be added directly to the developer to stop the development. In any case, the development does not stop immediately but continues for approximately 5 minutes after adding the Stop Solution.

Wash the gel several time in Destain II and finally with water. Store in water.

For gel drying, add 1.5% glycerol to the storage water.

VITA

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Publication

1. Suttipanta, N. and De-Eknamkul, W. 1998. Purification and characterization of deacetylipecoside synthase from the leaves of *Alangium salviifolium* Wang. Phytochemistry submitted.