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(54) Title: AMINOTRANSFERASE AND OXIDOREDUCTASE NUCLEIC ACIDS AND POLYPEPTIDES AND METHODS OF USING

(57) Abstract: The invention provides for aminotransferase and oxidoreductase polypeptides and nucleic acids encoding such polypeptides. Also provided are methods of using such aminotransferase and oxidoreductase nucleic acids and polypeptides.

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AMINOTRANSFERASE AND OXIDOREDUCTASE NUCLEIC ACIDS AND POLYPEPTIDES AND METHODS OF USING

INCORPORATION BY REFERENCE

A Sequence Listing is being filed concurrently with the filing of this application under PCT AI § 801(a). The accompanying Sequence Listing, identified as 07_0245WO01seq.txt, is herein incorporated by reference.

Appendix I is being filed concurrently with the filing of this application under PCT AI § 801(a). The accompanying Appendix, identified as 07_0245WO01app.txt, is a table related to the Sequence Listing and is herein incorporated by reference.

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims benefit of priority under 35 U.S.C. 119(e) to U.S. Application No. 61/018,814 filed January 3, 2008, which is incorporated herein by reference in its entirety.

TECHNICAL FIELD

This invention relates to nucleic acids and polypeptides, and more particularly to nucleic acids and polypeptides encoding aminotransferases and oxidoreductases as well as methods of using such aminotransferases and oxidoreductases.

BACKGROUND

An aminotransferase enzyme catalyzes a transamination reaction between an amino acid and an alpha-keto acid. Alpha-aminotransferases catalyze a reaction that removes the amino group from an amino acid, forming an alpha-keto acid, and transferring the amino group to a reactant α-keto acid, converting the keto acid into an amino acid. Therefore, an aminotransferase is useful in the production of amino acids.

An oxidoreductase enzyme such as a dehydrogenase catalyzes a reaction that oxidizes a substrate by transferring one or more protons and a pair of electrons to an acceptor (e.g., transfers electrons from a reductant to an oxidant). Therefore, an oxidoreductase is useful in catalyzing the oxidative deamination of amino acids to keto acids or the reductive amination of keto acids to amino acids.

SUMMARY

This disclosure provides for a number of different aminotransferase and oxidoreductase polypeptides and the nucleic acids encoding such aminotransferase and oxidoreductase polypeptides. This disclosure also provides for methods of using such aminotransferase and oxidoreductase nucleic acids and polypeptides.

In one aspect, the invention provides for methods of converting tryptophan to indole-3-pyruvate (or, alternatively, indole-3-pyruvate to tryptophan). Such methods include combining tryptophan (or indole-3-pyruvate) with a) one or more nucleic acid molecules chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 319, 321, 323, 325, 327, 329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, 357, 359, 361, 363, 365, 367, 369, 371, 373, 375, 377, 379, 381, 383, 385, 387, 389, 391, 393, 395, 397, 399, 401, 403, 405, 407, 409, 411, 413, 415, 417, 419, 421, 423, 425, 427, 429, 431, 433, 435, 437, 439, 441, 443, 445, 447, 449, 451, 453, 455, 457, 459, 461, 463, 465, 467, 469, 471, 473, 475, 477, 479, 481, 483, 485, 487, 489, 491, 493, 495, 497, 499, 501, 503, 505, 507, 509, 511, 513, 515, 517, 519, 521, 523, 525, 527, 529, 531, 533, 535, 537, 539, 541, 543, 545, 547, 549, 551, 553, 555, 557, 559, 561, 563, 565, 567, 569, 571, 573, 575, 577, 579, 581, 583, 585, 587 589, 591, 593, 595, 597, 599, 601, 603, 605, 607, 609, 611, 613, 615, 617, 619, 621, 623, 625, 627, 629, 631, 633, 635, 637, 639, 641, 643, 645, 647, 649, 651, 653, 655, 657, 659, 661, 663, 665, 667, 669, 671, 673, 675, 677, 679, 681, 683, 685, 687, 689, 691, 693, 695, 697, 699, 701, 703, 705, 707, 709, 711, 713, 715, 716, 719, 721, 723, 725, 727, 729, 731, 733, 735, 737, 739, 741, 743, 745, 747, 749, 751, 753, 755, 757, 759, 761, 763, 765, 767, 769, 771, 773, 775, 777, 779, 781, 783, 785, 787, 789, 791, 793, 795, 797, 799, 801, 803, 805, 807, 809, 811, 813, 815, 817, 819, 821, 823, 825, 827, 829, 831, 833, 835, 837, 839, 841, 843, 845, 847, 849, 851, 853, 855, 857, 859, 861, 863, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 961, 963, 965, 967, 969, 971, 973, and 975, wherein the one or more nucleic acid molecules encode polypeptides having aminotransferase (AT) or oxidoreductase activity; b) a variant of a), wherein

the variant encodes a polypeptide having AT or oxidoreductase activity; c) a fragment of a) or b), wherein the fragment encodes a polypeptide having AT or oxidoreductase activity; d) one or more polypeptides chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572, 574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 962, 964, 966, 968, 970, 972, 974, 976, 1069, 1070, 1071, 1072 and 1073, wherein the one or more polypeptides has AT or oxidoreductase activity; e) a variant of d), wherein the variant has AT or oxidoreductase activity; or f) a fragment of d) or e), wherein the fragment has AT or oxidoreductase activity.

In another aspect, the invention provides methods of converting MP to monatin (or, alternatively, monatin to MP). Such methods generally include combining MP (or monatin) with a) one or more nucleic acid molecules chosen from the group consisting of SEQ ID NOs:1, 3, 5,

7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 319, 321, 323, 325, 327, 329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, 357, 359, 361, 363, 365, 367, 369, 371, 373, 375, 377, 379, 381, 383, 385, 387, 389, 391, 393, 395, 397, 399, 401, 403, 405, 407, 409, 411, 413, 415, 417, 419, 421, 423, 425, 427, 429, 431, 433, 435, 437, 439, 441, 443, 445, 447, 449, 451, 453, 455, 457, 459, 461, 463, 465, 467, 469, 471, 473, 475, 477, 479, 481, 483, 485, 487, 489, 491, 493, 495, 497, 499, 501, 503, 505, 507, 509, 511, 513, 515, 517, 519, 521, 523, 525, 527, 529, 531, 533, 535, 537, 539, 541, 543, 545, 547, 549, 551, 553, 555, 557, 559, 561, 563, 565, 567, 569, 571, 573, 575, 577, 579, 581, 583, 585, 587 589, 591, 593, 595, 597, 599, 601, 603, 605, 607, 609, 611, 613, 615, 617, 619, 621, 623, 625, 627, 629, 631, 633, 635, 637, 639, 641, 643, 645, 647, 649, 651, 653, 655, 657, 659, 661, 663, 665, 667, 669, 671, 673, 675, 677, 679, 681, 683, 685, 687, 689, 691, 693, 695, 697, 699, 701, 703, 705, 707, 709, 711, 713, 715, 716, 719, 721, 723, 725, 727, 729, 731, 733, 735, 737, 739, 741, 743, 745, 747, 749, 751, 753, 755, 757, 759, 761, 763, 765, 767, 769, 771, 773, 775, 777, 779, 781, 783, 785, 787, 789, 791, 793, 795, 797, 799, 801, 803, 805, 807, 809, 811, 813, 815, 817, 819, 821, 823, 825, 827, 829, 831, 833, 835, 837, 839, 841, 843, 845, 847, 849, 851, 853, 855, 857, 859, 861, 863, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 961, 963, 965, 967, 969, 971, 973, and 975, wherein the one or more nucleic acid molecules encode polypeptides having aminotransferase (AT) or oxidoreductase activity; b) a variant of a), wherein the variant encodes a polypeptide having AT or oxidoreductase activity; c) a fragment of a) or b), wherein the fragment encodes a polypeptide having AT or oxidoreductase activity; d) one or more polypeptides chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of

the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572, 574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 962, 964, 966, 968, 970, 972, 974, 976, 1069, 1070, 1071, 1072 and 1073, wherein the one or more polypeptides has AT or oxidoreductase activity; e) a variant of d), wherein the variant has AT or oxidoreductase activity; or f) a fragment of d) or e), wherein the fragment has AT or oxidoreductase activity.

In one embodiment, the one or more nucleic acid molecules are chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 969, 971, 973, and 975. In another embodiment, the one or more polypeptides are chosen from the group consisting of SEQ ID

NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, and 976.

In certain embodiments, the nucleic acid molecule has a sequence selected from the group consisting of SEQ ID NOs:945, 891, 893, 219, 175, 1063, 1065, and 1067. In certain embodiments, the polypeptide has a sequence selected from the group consisting of SEQ ID NOs:946, 892, 894, 220, 176, 1064, 1066, and 1068. In certain embodiments, the polypeptide has a sequence that corresponds to the consensus sequence shown in SEQ ID NO:1069 or 1070. In certain embodiments, the polypeptide has a sequence that corresponds to the consensus sequence shown in SEQ ID NO:1071, 1072, and 1073.

In some embodiments, the variant is a nucleic acid molecule that has at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 99%) sequence identity to SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 969, 971, 973, and 975.

In some embodiments, the variant is a polypeptide that has at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 99%) sequence identity to SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N,

220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, and 976.

In one embodiment, the variant is a polypeptide that has at least 65% (e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99%) sequence identity to SEQ ID NO:220. In another embodiment, the variant is a polypeptide that has at least 80% (e.g., at least 85%, at least 90%, at least 95%, or at least 99%) sequence identity to SEQ ID NO:870. In yet another embodiment, the variant is a polypeptide that has at least 65% (e.g., at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99%) sequence identity to SEQ ID NO:894.

In certain embodiments, the variant is a mutant. Representative mutants include, without limitation, a mutant having a mutation at the residue that aligns with residue 243 of DAT 4978 (e.g., SEQ ID NO:870 T242N, SEQ ID NO:220 G240N, or SEQ ID NO:220 T241N). In one embodiment, the variant is a nucleic acid molecule that has been codon optimized. In certain embodiments, the variant polypeptide is a chimeric polypeptide.

In certain embodiments, a nucleic acid molecule is contained within an expression vector and can be, for example, overexpressed. In certain embodiments, the aminotransferase or oxidoreductase polypeptide is immobilized on a solid support. In certain embodiments, the tryptophan or the MP is a substituted tryptophan or a substituted MP. A representative tryptophan is 6-chloro-D-tryptophan.

In another aspect, the invention provides methods of converting tryptophan to indole-3-pyruvate (or, alternatively, indole-3-pyruvate to tryptophan). Such methods typically include combining tryptophan (or indole-3-pyruvate) with a) one or more nucleic acid molecules chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 969, 971, 973, and 975, wherein the one or more nucleic acid molecules encode polypeptides having D-

aminotransferase (DAT) activity; b) a variant of a), wherein the variant encodes a polypeptide having DAT activity; c) a fragment of a) or b), wherein the fragment encodes a polypeptide having DAT activity; d) one or more polypeptides chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, 976, 1069, 1070, 1071, 1072 and 1073, wherein the one or more polypeptides has DAT activity; e) a variant of d), wherein the variant has DAT activity; or f) a fragment of d) or e), wherein the fragment has DAT activity.

In still another aspect, the invention provides for methods of converting MP to monatin (or, alternatively, monatin to MP). Such methods generally include combining MP (or monatin) with a) one or more nucleic acid molecules chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 969, 971, 973, and 975, wherein the one or more nucleic acid molecules encode polypeptides having DAT activity; b) a variant of a), wherein the variant encodes a polypeptide having DAT activity; c) a fragment of a) or b), wherein the fragment encodes a polypeptide having DAT activity; d) one or more polypeptides chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170,

172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, 976, 1069, 1070, 1071, 1072 and 1073, wherein the one or more polypeptides has DAT activity; e) a variant of d), wherein the variant has DAT activity; or f) a fragment of d) or e), wherein the fragment has DAT activity.

In certain embodiments, the nucleic acid molecule or polypeptide has a sequence selected from the group consisting of SEQ ID NO:945, 891, 893, 219, 175, 1063, 1065, and 1067. In certain embodiments, the polypeptide has a sequence that corresponds to a consensus sequence shown in SEQ ID NO:1069, 1070, 1071, 1072 or 1073. In some embodiments, the tryptophan or the MP is a substituted tryptophan or a substituted MP. A representative substituted tryptophan is 6-chloro-D-tryptophan.

In still another aspect, the invention provides methods of making monatin. Generally, such methods include contacting tryptophan, under conditions in which monatin is produced, with a C3 carbon source and a) one or more nucleic acid molecules chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 969, 971, 973, and 975, wherein the one or more nucleic acid molecules encode polypeptides having D-aminotransferase (DAT) activity; b) a variant of a), wherein the variant encodes a polypeptide having DAT activity; c) a fragment of a) or b), wherein the fragment encodes a polypeptide having DAT activity; d) one or more polypeptides chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190,

192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, 976, 1069, 1070, 1071, 1072 and 1073, wherein the one or more polypeptides has DAT activity; e) a variant of d), wherein the variant has DAT activity; or f) a fragment of d) or e), wherein the fragment has DAT activity.

In some embodiments, the nucleic acid molecule is chosen from the group consisting of SEQ ID NO: 945, 891, 893, 219, 175, 1063, 1065, and 1067. In some embodiments, the C3 carbon source is selected from the group consisting of pyruvate, oxaloacetate, and serine. In some embodiments, the method further comprises adding or including a synthase / lyase (EC 4.1.2.- or 4.1.3.-) polypeptide. Representative synthase / lyase (EC 4.1.2.- or 4.1.3.-) polypeptides include aldolases such as KHG aldolases (EC 4.1.3.16) or HMG aldolases (EC 4.1.3.16).

In some embodiments of the above-indicated methods, the monatin produced is R,R monatin. In some embodiments, the monatin produced is S,R monatin. In certain embodiments, the tryptophan is a substituted tryptophan such as, for example, 6-chloro-D-tryptophan.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the drawings and detailed description, and from the claims.

DESCRIPTION OF DRAWINGS

Figure 1 is an alignment of SEQ ID NO:894, 1066, 1064, and 1068.

Figure 2 is an alignment of SEQ ID NO:870, 910, and several *Bacillus* sequences. Consensus sequences A and B (SEQ ID NO:1069 and 1070, respectively) directed toward the novel portions of SEQ ID NO:870 were developed from this alignment.

Figure 3 is an alignment of SEQ ID NO:946, 894, 892, 220, 176, 1064, 1066, and 1068. Consensus sequences C, D and E (SEQ ID NO:1071, 1072 and 1073, respectively) were developed from this alignment.

Figure 4 is a model of 3DAA-D-amino acid aminotransferase, with numbered residues indicating those sites selected for TMCA^{SM} evolution, as described in detail below.

Like reference symbols in the various drawings indicate like elements.

DETAILED DESCRIPTION

Disclosed herein are a number of different nucleic acid molecules encoding polypeptides having aminotransferase (AT) activity (e.g., transaminase activity). Specifically disclosed are a number of D-aminotransferases (DATs). DATs catalyze a transamination reaction (e.g., D-alanine + 2-oxoglutarate <=> pyruvate + D-glutamate). Also provided are a number of different nucleic acid molecules encoding polypeptides having oxidoreductase activity (e.g., dehydrogenase activity). Oxidoreductases such as dehydrogenases catalyze an oxidation-reduction reaction (e.g., D-amino acid + H_2O + acceptor <=> a 2-oxo acid + NH_3 + reduced acceptor). The nucleic acids or polypeptides disclosed herein can be used to convert tryptophan to indole-3-pyruvate and/or 2-hydroxy 2-(indol-3ylmethyl)-4-keto glutaric acid ("monatin precursor" or "MP") to monatin.

Isolated Nucleic Acid Molecules and Purified Polypeptides

The present invention is based, in part, on the identification of nucleic acid molecules encoding polypeptides having aminotransferase (AT) activity, herein referred to as "AT" nucleic acid molecules or polypeptides, where appropriate. The present invention also is based, in part, on the identification of nucleic acid molecules encoding polypeptides having oxidoreductase activity, herein referred to as "oxidoreductase" nucleic acid molecules or polypeptides, where appropriate.

Particular nucleic acid molecules described herein include the sequences shown in SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 319, 321, 323, 325, 327,

329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, 357, 359, 361, 363, 365, 367, 369, 371, 373, 375, 377, 379, 381, 383, 385, 387, 389, 391, 393, 395, 397, 399, 401, 403, 405, 407, 409, 411, 413, 415, 417, 419, 421, 423, 425, 427, 429, 431, 433, 435, 437, 439, 441, 443, 445, 447, 449, 451, 453, 455, 457, 459, 461, 463, 465, 467, 469, 471, 473, 475, 477, 479, 481, 483, 485, 487, 489, 491, 493, 495, 497, 499, 501, 503, 505, 507, 509, 511, 513, 515, 517, 519, 521, 523, 525, 527, 529, 531, 533, 535, 537, 539, 541, 543, 545, 547, 549, 551, 553, 555, 557, 559, 561, 563, 565, 567, 569, 571, 573, 575, 577, 579, 581, 583, 585, 587 589, 591, 593, 595, 597, 599, 601, 603, 605, 607, 609, 611, 613, 615, 617, 619, 621, 623, 625, 627, 629, 631, 633, 635, 637, 639, 641, 643, 645, 647, 649, 651, 653, 655, 657, 659, 661, 663, 665, 667, 669, 671, 673, 675, 677, 679, 681, 683, 685, 687, 689, 691, 693, 695, 697, 699, 701, 703, 705, 707, 709, 711, 713, 715, 716, 719, 721, 723, 725, 727, 729, 731, 733, 735, 737, 739, 741, 743, 745, 747, 749, 751, 753, 755, 757, 759, 761, 763, 765, 767, 769, 771, 773, 775, 777, 779, 781, 783, 785, 787, 789, 791, 793, 795, 797, 799, 801, 803, 805, 807, 809, 811, 813, 815, 817, 819, 821, 823, 825, 827, 829, 831, 833, 835, 837, 839, 841, 843, 845, 847, 849, 851, 853, 855, 857, 859, 861, 863, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 961, 963, 965, 967, 969, 971, 973, and 975. As used herein, the term "nucleic acid molecule" can include DNA molecules and RNA molecules, analogs of DNA or RNA generated using nucleotide analogs. A nucleic acid molecule of the invention can be single-stranded or double-stranded, depending upon its intended use. Nucleic acid molecules of the invention include molecules that have at least 75% sequence identity (*e.g.,* at least 80%, 85%, 90%, 95%, or 99% sequence identity) to any of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 319, 321, 323, 325, 327, 329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, 357, 359, 361, 363, 365, 367, 369, 371, 373, 375, 377, 379, 381, 383, 385, 387, 389, 391, 393, 395, 397, 399, 401, 403, 405, 407, 409, 411, 413, 415, 417, 419, 421, 423, 425, 427, 429, 431, 433, 435, 437, 439, 441, 443, 445, 447, 449, 451, 453, 455, 457, 459, 461, 463, 465, 467, 469, 471, 473, 475, 477, 479, 481, 483, 485, 487, 489, 491, 493, 495, 497, 499, 501, 503, 505, 507, 509, 511, 513, 515, 517,

519, 521, 523, 525, 527, 529, 531, 533, 535, 537, 539, 541, 543, 545, 547, 549, 551, 553, 555, 557, 559, 561, 563, 565, 567, 569, 571, 573, 575, 577, 579, 581, 583, 585, 587 589, 591, 593, 595, 597, 599, 601, 603, 605, 607, 609, 611, 613, 615, 617, 619, 621, 623, 625, 627, 629, 631, 633, 635, 637, 639, 641, 643, 645, 647, 649, 651, 653, 655, 657, 659, 661, 663, 665, 667, 669, 671, 673, 675, 677, 679, 681, 683, 685, 687, 689, 691, 693, 695, 697, 699, 701, 703, 705, 707, 709, 711, 713, 715, 716, 719, 721, 723, 725, 727, 729, 731, 733, 735, 737, 739, 741, 743, 745, 747, 749, 751, 753, 755, 757, 759, 761, 763, 765, 767, 769, 771, 773, 775, 777, 779, 781, 783, 785, 787, 789, 791, 793, 795, 797, 799, 801, 803, 805, 807, 809, 811, 813, 815, 817, 819, 821, 823, 825, 827, 829, 831, 833, 835, 837, 839, 841, 843, 845, 847, 849, 851, 853, 855, 857, 859, 861, 863, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 961, 963, 965, 967, 969, 971, 973, and 975 and that have functional AT or oxidoreductase activity.

In calculating percent sequence identity, two sequences are aligned and the number of identical matches of nucleotides or amino acid residues between the two sequences is determined. The number of identical matches is divided by the length of the aligned region (*i.e.*, the number of aligned nucleotides or amino acid residues) and multiplied by 100 to arrive at a percent sequence identity value. It will be appreciated that the length of the aligned region can be a portion of one or both sequences up to the full-length size of the shortest sequence. It will be appreciated that a single sequence can align differently with other sequences and hence, can have different percent sequence identity values over each aligned region. It is noted that the percent identity value is usually rounded to the nearest integer. For example, 78.1%, 78.2%, 78.3%, and 78.4% are rounded down to 78%, while 78.5%, 78.6%, 78.7%, 78.8%, and 78.9% are rounded up to 79%. It is also noted that the length of the aligned region is always an integer.

The alignment of two or more sequences to determine percent sequence identity is performed using the algorithm described by Altschul et al. (1997, *Nucleic Acids Res.*, 25:3389-3402) as incorporated into BLAST (basic local alignment search tool) programs, available at ncbi.nlm.nih.gov on the World Wide Web. BLAST searches can be performed to determine percent sequence identity between an AT nucleic acid molecule described herein and any other sequence or portion thereof aligned using the Altschul et al. algorithm. BLASTN is the program used to align and compare the identity between nucleic acid sequences, while BLASTP is the program used to align and compare the identity between amino acid sequences. When utilizing BLAST programs to calculate the percent identity between a sequence of the invention and another sequence, the default parameters of the respective programs are used.

Nucleic acid molecules of the invention, for example, those between about 10 and about 50 nucleotides in length, can be used, under standard amplification conditions, to amplify an AT or oxidoreductase nucleic acid molecule. Amplification of an AT or oxidoreductase nucleic acid can be for the purpose of detecting the presence or absence of an AT or oxidoreductase nucleic acid molecule or for the purpose of obtaining (e.g., cloning) an AT or oxidoreductase nucleic acid molecule. As used herein, standard amplification conditions refer to the basic components of an amplification reaction mix, and cycling conditions that include multiple cycles of denaturing the template nucleic acid, annealing the oligonucleotide primers to the template nucleic acid, and extension of the primers by the polymerase to produce an amplification product (see, for example, U.S. Patent Nos. 4,683,195; 4,683,202; 4,800,159; and 4,965,188). The basic components of an amplification reaction mix generally include, for example, each of the four deoxynucleoside triphosphates, (*e.g.,* dATP, dCTP, dTTP, and dGTP, or analogs thereof), oligonucleotide primers, template nucleic acid, and a polymerase enzyme. Template nucleic acid is typically denatured at a temperature of at least about 90°C, and extension from primers is typically performed at a temperature of at least about 72°C. In addition, variations to the original PCR methods (e.g., anchor PCR, RACE PCR, or ligation chain reaction (LCR)) have been developed and are known in the art. See, for example, Landegran et al., 1988, *Science*, 241:1077-1080; and Nakazawa et al., 1994, *Proc. Natl. Acad. Sci. USA*, 91:360-364).

The annealing temperature can be used to control the specificity of amplification. The temperature at which primers anneal to template nucleic acid must be below the Tm of each of the primers, but high enough to avoid non-specific annealing of primers to the template nucleic acid. The Tm is the temperature at which half of the DNA duplexes have separated into single strands, and can be predicted for an oligonucleotide primer using the formula provided in section 11.46 of Sambrook et al. (1989, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). Non-specific amplification products are detected as bands on a gel that are not the size expected for the correct amplification product.

Nucleic acid molecules of the invention, for example, those between about 10 and several hundred nucleotides in length (up to several thousand nucleotides in length), can be used, under standard hybridization conditions, to hybridize to an AT or oxidoreductase nucleic acid molecule. Hybridization to an AT or oxidoreductase nucleic acid molecule can be for the purpose of detecting or obtaining an AT or oxidoreductase nucleic acid molecule. As used herein, standard hybridization conditions between nucleic acid molecules are discussed in detail in Sambrook et al. (1989, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY; Sections 7.37-7.57, 9.47-9.57, 11.7-11.8, and 11.45-11.57). For oligonucleotide probes less than about 100 nucleotides, Sambrook et al.

discloses suitable Southern blot conditions in Sections 11.45-11.46. The Tm between a sequence that is less than 100 nucleotides in length and a second sequence can be calculated using the formula provided in Section 11.46. Sambrook et al. additionally discloses prehybridization and hybridization conditions for a Southern blot that uses oligonucleotide probes greater than about 100 nucleotides (see Sections 9.47-9.52). Hybridizations with an oligonucleotide greater than 100 nucleotides generally are performed 15-25°C below the Tm. The Tm between a sequence greater than 100 nucleotides in length and a second sequence can be calculated using the formula provided in Sections 9.50-9.51 of Sambrook et al. Additionally, Sambrook et al. recommends the conditions indicated in Section 9.54 for washing a Southern blot that has been probed with an oligonucleotide greater than about 100 nucleotides.

The conditions under which membranes containing nucleic acids are prehybridized and hybridized, as well as the conditions under which membranes containing nucleic acids are washed to remove excess and non-specifically bound probe can play a significant role in the stringency of the hybridization. For example, hybridization and washing may be carried out under conditions of low stringency, moderate stringency or high stringency. Such conditions are described, for example, in Sambrook et al. section 11.45-11.46. The conditions used to achieve a particular level of stringency will vary, depending on the nature of the nucleic acids being hybridized. For example, the length, degree of complementarity, nucleotide sequence composition (*e.g.*, G/C vs. A/T nucleotide content) and nucleic acid type (*e.g.*, RNA v. DNA) of the hybridizing regions of the nucleic acids can be considered in selecting hybridization conditions. For example, washing conditions can be made more stringent by decreasing the salt concentration in the wash solutions and/or by increasing the temperature at which the washes are performed.

The amount of hybridization can be quantitated directly on a membrane or from an autoradiograph using, for example, a PhosphorImager or a Densitometer (Molecular Dynamics, Sunnyvale, CA). It is understood by those of skill in the art that interpreting the amount of hybridization can be affected by, for example, the specific activity of the labeled oligonucleotide probe, the number of probe-binding sites on the target nucleic acid, and the amount of exposure of an autoradiograph or other detection medium. It will be readily appreciated that, although any number of hybridization, washing and detection conditions can be used to examine hybridization of a probe nucleic acid molecule to immobilized target nucleic acids, it is more important to examine hybridization of a probe to target nucleic acids under identical hybridization, washing, and detection conditions. Preferably, the target nucleic acids are on the same membrane. It can be appreciated by those of skill in the art that appropriate positive and negative controls should

be performed with every set of amplification or hybridization reactions to avoid uncertainties related to contamination and/or non-specific annealing of oligonucleotide primers or probes.

Oligonucleotide primers or probes specifically anneal or hybridize to one or more AT or oxidoreductase nucleic acids. For amplification, a pair of oligonucleotide primers generally anneal to opposite strands of the template nucleic acid, and should be an appropriate distance from one another such that the polymerase can effectively polymerize across the region and such that the amplification product can be readily detected using, for example, electrophoresis. Oligonucleotide primers or probes can be designed using, for example, a computer program such as OLIGO (Molecular Biology Insights Inc., Cascade, CO) to assist in designing oligonucleotides. Typically, oligonucleotide primers are 10 to 30 or 40 or 50 nucleotides in length (*e.g.*, 10, 11, 12, 13, 14, 15, 16, 17, 18,19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides in length), but can be longer or shorter if appropriate amplification conditions are used.

Non-limiting representative pairs of oligonucleotide primers that were used to amplify D-aminotransferase (DAT) nucleic acid molecules are shown in Tables 2-8 (SEQ ID NOs:978-1062 and 1074-1083), Table 46 (SEQ ID NOs:1084-1103) and Table 54 (SEQ ID NOs:1104-1125). The sequences shown in SEQ ID NOs:978-1062 and 1074-1083 are non-limiting examples of oligonucleotide primers that can be used to amplify AT nucleic acid molecules. Oligonucleotides in accordance with the invention can be obtained by restriction enzyme digestion of an AT or oxidoreductase nucleic acid molecules or can be prepared by standard chemical synthesis and other known techniques.

As used herein, an "isolated" nucleic acid molecule is a nucleic acid molecule that is separated from other nucleic acid molecules that are usually associated with the isolated nucleic acid molecule. Thus, an "isolated" nucleic acid molecule includes, without limitation, a nucleic acid molecule that is free of sequences that naturally flank one or both ends of the nucleic acid in the genome of the organism from which the isolated nucleic acid is derived (*e.g.*, a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease digestion). Such an isolated nucleic acid molecule is generally introduced into a vector (*e.g.*, a cloning vector, or an expression vector) for convenience of manipulation or to generate a fusion nucleic acid molecule. In addition, an isolated nucleic acid molecule can include an engineered nucleic acid molecule such as a recombinant or a synthetic nucleic acid molecule. A nucleic acid molecule existing among hundreds to millions of other nucleic acid molecules within, for example, a nucleic acid library (*e.g.*, a cDNA, or genomic library) or a portion of a gel (*e.g.*, agarose, or polyacrylamine) containing restriction-digested genomic DNA is not to be considered an isolated nucleic acid.

Isolated nucleic acid molecules described herein having AT or oxidoreductase activity can be obtained using techniques routine in the art, many of which are described in the Examples herein. For example, isolated nucleic acids within the scope of the invention can be obtained using any method including, without limitation, recombinant nucleic acid technology, the polymerase chain reaction (e.g., PCR, e.g., direct amplification or site-directed mutagenesis), and/or nucleic acid hybridization techniques (e.g., Southern blotting). General PCR techniques are described, for example in *PCR Primer: A Laboratory Manual*, Dieffenbach & Dveksler, Eds., Cold Spring Harbor Laboratory Press, 1995. Recombinant nucleic acid techniques include, for example, restriction enzyme digestion and ligation, which can be used to isolate an AT or oxidoreductase nucleic acid molecule as described herein. Isolated nucleic acids of the invention also can be chemically synthesized, either as a single nucleic acid molecule or as a series of oligonucleotides.

Techniques for the manipulation of nucleic acids, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization, amplification and the like are well described in the scientific and patent literature, see, e.g., Sambrook et al., Eds., 1989, *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Vols 1-3, Cold Spring Harbor Laboratory; *Current Protocols in Molecular Biology*, 1997, Ausubel, Ed. John Wiley & Sons, Inc., New York; *Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization with Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation*, Tijssen, Ed. Elsevier, N.Y. (1993).

Purified AT or oxidoreductase polypeptides, as well as polypeptide fragments having AT or oxidoreductase activity, are within the scope of the invention. AT polypeptides refer to polypeptides that catalyze a reaction between an amino group and a keto acid. Specifically, a transamination reaction by a DAT involves removing an amino group from an amino acid leaving behind an alpha-keto acid, and transferring the amino group to the reactant alpha-keto acid, thereby converting the alpha-keto acid into an amino acid. The predicted amino acid sequences of AT polypeptides are shown in SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926,

928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, and 976. An oxidoreductase polypeptide refers to a polypeptide that catalyzes an oxidation-reduction reaction, and the predicted amino acid sequences of oxidoreductase polypeptides are shown in SEQ ID NOs:252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572, 574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 962, 964, 966 and 968.

The term "purified" polypeptide as used herein refers to a polypeptide that has been separated from cellular components that naturally accompany it. Typically, a polypeptide is considered "purified" when it is at least partically free from the proteins and naturally occurring molecules with which it is naturally associated. The extent of enrichment or purity of an AT or oxidoreductase polypeptide can be measured using any appropriate method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

The invention also provides for AT and oxidoreductase polypeptides that differ in sequence from SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286,

288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572, 574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 866, 868, 870, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 962, 964, 966, 968, 970, 972, 974, and 976. For example, the skilled artisan will appreciate that changes can be introduced into an AT or oxidoreductase polypeptide (e.g., SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572,

574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 866, 868, 870, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 962, 964, 966, 968, 970, 972, 974, and 976) or in an AT or oxidoreductase nucleic acid molecule (e.g., SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 319, 321, 323, 325, 327, 329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, 357, 359, 361, 363, 365, 367, 369, 371, 373, 375, 377, 379, 381, 383, 385, 387, 389, 391, 393, 395, 397, 399, 401, 403, 405, 407, 409, 411, 413, 415, 417, 419, 421, 423, 425, 427, 429, 431, 433, 435, 437, 439, 441, 443, 445, 447, 449, 451, 453, 455, 457, 459, 461, 463, 465, 467, 469, 471, 473, 475, 477, 479, 481, 483, 485, 487, 489, 491, 493, 495, 497, 499, 501, 503, 505, 507, 509, 511, 513, 515, 517, 519, 521, 523, 525, 527, 529, 531, 533, 535, 537, 539, 541, 543, 545, 547, 549, 551, 553, 555, 557, 559, 561, 563, 565, 567, 569, 571, 573, 575, 577, 579, 581, 583, 585, 587 589, 591, 593, 595, 597, 599, 601, 603, 605, 607, 609, 611, 613, 615, 617, 619, 621, 623, 625, 627, 629, 631, 633, 635, 637, 639, 641, 643, 645, 647, 649, 651, 653, 655, 657, 659, 661, 663, 665, 667, 669, 671, 673, 675, 677, 679, 681, 683, 685, 687, 689, 691, 693, 695, 697, 699, 701, 703, 705, 707, 709, 711, 713, 715, 716, 719, 721, 723, 725, 727, 729, 731, 733, 735, 737, 739, 741, 743, 745, 747, 749, 751, 753, 755, 757, 759, 761, 763, 765, 767, 769, 771, 773, 775, 777, 779, 781, 783, 785, 787, 789, 791, 793, 795, 797, 799, 801, 803, 805, 807, 809, 811, 813, 815, 817, 819, 821, 823, 825, 827, 829, 831, 833, 835, 837, 839, 841, 843, 845, 847, 849, 851, 853, 855, 857, 859, 861, 863, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923,

925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 961, 963, 965, 967, 969, 971, 973, and 975), thereby leading to changes in the amino acid sequence of the encoded polypeptide. AT and oxidoreductase polypeptides that differ in sequence from SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572, 574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 866, 868, 870, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 962, 964, 966, 968, 970, 972, 974, and 976 and that retain aminotransferase and oxidoreductase activity, respectively, readily can be identified by screening methods routinely used in the art.

For example, changes can be introduced into an AT or oxidoreductase nucleic acid coding sequence that lead to conservative and/or non-conservative amino acid substitutions at one or more amino acid residues in the encoded AT or oxidoreductase polypeptide. Changes in nucleic acid sequences can be generated by standard techniques, such as site-directed

mutagenesis, PCR-mediated mutagenesis of a nucleic acid encoding such a polypeptide, or directed evolution. In addition, changes in the polypeptide sequence can be introduced randomly along all or part of the AT or oxidoreductase polypeptide, such as by saturation mutagenesis of the corresponding nucleic acid. Alternatively, changes can be introduced into a nucleic acid or polypeptide sequence by chemically synthesizing a nucleic acid molecule or polypeptide having such changes.

A "conservative amino acid substitution" is one in which one amino acid residue is replaced with a different amino acid residue having a similar side chain. Similarity between amino acid residues has been assessed in the art. For example, Dayhoff et al. (1978, in *Atlas of Protein Sequence and Structure*, 5(Suppl. 3):345-352) provides frequency tables for amino acid substitutions that can be employed as a measure of amino acid similarity. Examples of conservative substitutions include, for example, replacement of an aliphatic amino acid with another aliphatic amino acid; replacement of a serine with a threonine or vice versa; replacement of an acidic residue with another acidic residue; replacement of a residue bearing an amide group with another residue bearing an amide group; exchange of a basic residue with another basic residue; or replacement of an aromatic residue with another aromatic residue. A non-conservative substitution is one in which an amino acid residue is replaced with an amino acid residue that does not have a similar side chain.

Changes in a nucleic acid can be introduced using one or more mutagens. Mutagens include, without limitation, ultraviolet light, gamma irradiation, or chemical mutagens (e.g., mitomycin, nitrous acid, photoactivated psoralens, sodium bisulfite, nitrous acid, hydroxylamine, hydrazine or formic acid). Other mutagens are analogues of nucleotide precursors, e.g., nitrosoguanidine, 5-bromouracil, 2-aminopurine, or acridine. Intercalating agents such as proflavine, acriflavine, quinacrine and the like can also be used.

Changes also can be introduced into an AT or oxidoreductase nucleic acid and/or polypeptide by methods such as error-prone PCR, shuffling, oligonucleotide-directed mutagenesis, assembly PCR, sexual PCR mutagenesis, *in vivo* mutagenesis, cassette mutagenesis, recursive ensemble mutagenesis, exponential ensemble mutagenesis, gene reassembly (e.g., GeneReassembly, see, e.g., U.S. Patent No. 6,537,776), Gene Site Saturation Mutagenesis (GSSM), synthetic ligation reassembly (SLR), or a combination thereof. Changes also can be introduced into polypeptides by methods such as recombination, recursive sequence recombination, phosphothioate-modified DNA mutagenesis, uracil-containing template mutagenesis, gapped duplex mutagenesis, point mismatch repair mutagenesis, repair-deficient host strain mutagenesis, chemical mutagenesis, radiogenic mutagenesis, deletion mutagenesis,

restriction-selection mutagenesis, restriction-purification mutagenesis, artificial gene synthesis, ensemble mutagenesis, chimeric nucleic acid multimer creation, or any combination thereof.

An AT or oxidoreductase nucleic acid can be codon optimized if so desired. For example, a non-preferred or a less preferred codon can be identified and replaced with a preferred or neutrally used codon encoding the same amino acid as the replaced codon. A preferred codon is a codon over-represented in coding sequences in genes in the host cell and a non-preferred or less preferred codon is a codon under-represented in coding sequences in genes in the host cell, thereby modifying the nucleic acid to increase its expression in a host cell. An AT or oxidoreductase nucleic acid can be optimized for particular codon usage from any host cell (e.g., any of the host cells described herein). See, for example, U.S. Patent No. 5,795,737 for a representative description of codon optimization. In addition to codon optimization, a nucleic acid can undergo directed evolution. See, for example, U.S. Patent No. 6,361,974.

Other changes also are within the scope of this disclosure. For example, one, two, three, four or more amino acids can be removed from the carboxy- and/or amino- terminal ends of an aminotransferase or oxidoreductase polypeptide without significantly altering the biological activity. In addition, one or more amino acids can be changed to increase or decrease the pI of a polypeptide. In some embodiments, a residue can be changed to, for example, a glutamate. Also provided are chimeric aminotransferase or oxidoreductase polypeptides. For example, a chimeric AT or oxidoreductase polypeptide can include portions of different binding or catalytic domains. Methods of recombining different domains from different polypeptides and screening the resultant chimerics to find the best combination for a particular application or substrate are routine in the art.

One particular change in sequence that was exemplified herein involves the residue corresponding to residue 243 in a DAT from ATCC Accession No. 4978 (DAT 4978). In one instance, the polypeptide sequence of the SEQ ID NO:870 DAT was aligned with DAT 4978 and the residue in SEQ ID NO:870 that aligns with position 243 in DAT 4978 was identified (residue 242) and changed from Thr to Asn (SEQ ID NO:870 T242N). In another instance, the polypeptide sequence of the SEQ ID NO:220 DAT was aligned with DAT 4978 and the residue in SEQ ID NO:220 that aligns with position 243 in DAT 4978 was identified (either residue 240 or 241) and changed from Gly to Asn or Thr to Asn, respectively (SEQ ID NO:220 G240N and SEQ ID NO:220 T241N). Those of skill in the art can readily identify the residue that corresponds to residue 243 from DAT 4978 in any of the DATs disclosed herein and introduce a change into the polypeptide sequence at that particular residue. A number of additional DAT mutants were made and are listed in Tables 43 and 52.

It is noted that SEQ ID NO:894 is a novel DAT, for which the closest sequence in the public databases exhibits only 60% sequence identity to the SEQ ID NO:894 polypeptide. Therefore, polypeptides of the invention include sequences that have at least, for example, 65% sequence identity (e.g., at least 70%, 75%, 80%, 85%, 90%, 95%, or 99% sequence identity) to SEQ ID NO:894 and that have functional DAT activity. In addition, SEQ ID NO:870 also is a novel DAT and has 76% sequence identity to a *Bacillus* DAT polypeptide and 69% sequence identity to a *B. sphaericus* DAT polypeptide. Therefore, polypeptides of the invention include sequences having at least 80% sequence identity (e.g., at least 85%, 90%, 95%, or 99% sequence identity) to SEQ ID NO:870 and that have DAT activity. Further, SEQ ID NO:220 is a novel DAT that has 62% sequence identity to a DAT polypeptide from *C. beijerinckii*. Therefore, polypeptides of the invention include sequences having at least 65% sequence identity (e.g., at least 70%, 75%, 80%, 85%, 90%, 95%, or 99% sequence identity) to SEQ ID NO:220.

In one instance, SEQ ID NO:870 and 910 were aligned with published DATs and a consensus sequence was determined. The consensus sequence for SEQ ID NO:870-like DAT polypeptides that make it unique from the rest of the group of Bacillus-like DAT polypeptides is shown in SEQ ID NO:1069 (consensus sequence A). In another instance, SEQ ID NO:176, 220, 892, 894 and 946 were aligned and a consensus sequence was determined. The consensus sequence for this group of DATs is shown in SEQ ID NO:1071 (consensus sequence C). SEQ ID NO:1070 (consensus sequence B) represents a slightly more conservative consensus sequence relative to SEQ ID NO:1069 (consensus sequence A), while SEQ ID NO:1072 (consensus sequence D) and SEQ ID NO:1073 (consensus sequence E) correspond to slightly more conservative consensus sequences relative to SEQ ID NO:1071 (consensus sequence C). It is expected that polypeptides having a consensus sequence that corresponds to consensus sequence A, B, C, D or E as disclosed herein would exhibit DAT activity.

A fragment of an aminotransferase and oxidoreductase nucleic acid or polypeptide refers to a portion of a full-length aminotransferase and oxidoreductase nucleic acid or polypeptide. As used herein, "functional fragments" are those fragments of an aminotransferase or oxidoreductase polypeptide that retain the respective enzymatic activity. "Functional fragments" also refer to fragments of an aminotransferase or oxidoreductase nucleic acid that encode a polypeptide that retains the respective anzymatic activity. For example, functional fragments can be used in *in vitro* or *in vivo* reactions to catalyze transaminase or oxidation-reduction reactions, respectively.

AT or oxidoreductase polypeptides can be obtained (e.g., purified) from natural sources (*e.g.*, a biological sample) by known methods such as DEAE ion exchange, gel filtration, and hydroxyapatite chromatography. Natural sources include, but are not limited to, microorganisms

such as bacteria and yeast. A purified AT or oxidoreductase polypeptide also can be obtained, for example, by cloning and expressing an AT or oxidoreductase nucleic acid (e.g., SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 319, 321, 323, 325, 327, 329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, 357, 359, 361, 363, 365, 367, 369, 371, 373, 375, 377, 379, 381, 383, 385, 387, 389, 391, 393, 395, 397, 399, 401, 403, 405, 407, 409, 411, 413, 415, 417, 419, 421, 423, 425, 427, 429, 431, 433, 435, 437, 439, 441, 443, 445, 447, 449, 451, 453, 455, 457, 459, 461, 463, 465, 467, 469, 471, 473, 475, 477, 479, 481, 483, 485, 487, 489, 491, 493, 495, 497, 499, 501, 503, 505, 507, 509, 511, 513, 515, 517, 519, 521, 523, 525, 527, 529, 531, 533, 535, 537, 539, 541, 543, 545, 547, 549, 551, 553, 555, 557, 559, 561, 563, 565, 567, 569, 571, 573, 575, 577, 579, 581, 583, 585, 587 589, 591, 593, 595, 597, 599, 601, 603, 605, 607, 609, 611, 613, 615, 617, 619, 621, 623, 625, 627, 629, 631, 633, 635, 637, 639, 641, 643, 645, 647, 649, 651, 653, 655, 657, 659, 661, 663, 665, 667, 669, 671, 673, 675, 677, 679, 681, 683, 685, 687, 689, 691, 693, 695, 697, 699, 701, 703, 705, 707, 709, 711, 713, 715, 716, 719, 721, 723, 725, 727, 729, 731, 733, 735, 737, 739, 741, 743, 745, 747, 749, 751, 753, 755, 757, 759, 761, 763, 765, 767, 769, 771, 773, 775, 777, 779, 781, 783, 785, 787, 789, 791, 793, 795, 797, 799, 801, 803, 805, 807, 809, 811, 813, 815, 817, 819, 821, 823, 825, 827, 829, 831, 833, 835, 837, 839, 841, 843, 845, 847, 849, 851, 853, 855, 857, 859, 861, 863, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 961, 963, 965, 967, 969, 971, 973, and 975) and purifying the resultant polypeptide using, for example, any of the known expression systems including, but not limited to, glutathione S-transferase (GST), pGEX (Pharmacia Biotech Inc), pMAL (New England Biolabs, Beverly, MA) or pRIT5 (Pharmacia, Piscataway, NJ)). In addition, a purified AT or oxidoreductase polypeptide can be obtained by chemical synthesis using, for example, solid-phase synthesis techniques (see e.g., Roberge, 1995, *Science*, 269:202; Merrifield, 1997, *Methods Enzymol.*, 289:3-13).

A purified AT or oxidoreductase polypeptide or a fragment thereof can be used as an immunogen to generate polyclonal or monoclonal antibodies that have specific binding affinity

for one or more AT or oxidoreductase polypeptides. Such antibodies can be generated using standard techniques that are used routinely in the art. Full-length AT or oxidoreductase polypeptides or, alternatively, antigenic fragments of AT or oxidoreductase polypeptides can be used as immunogens. An antigenic fragment of an AT or oxidoreductase polypeptide usually includes at least 8 (*e.g.,* 10, 15, 20, or 30) amino acid residues of an AT or oxidoreductase polypeptide (e.g., having the sequence shown in SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572, 574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 866, 868, 870, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 962, 964, 966, 968, 970, 972, 974, and 976), and encompasses an epitope of an AT or oxidoreductase polypeptide such that an antibody (e.g., polyclonal or monoclonal; chimeric or humanized) raised against the antigenic fragment has specific binding affinity for one or more AT or oxidoreductase polypeptides.

Polypeptides can be detected and quantified by any method known in the art including, but not limited to, nuclear magnetic resonance (NMR), spectrophotometry, radiography (protein radiolabeling), electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, various immunological methods, e.g. immunoprecipitation, immunodiffusion, immuno-electrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immuno-fluorescent assays, gel electrophoresis (e.g., SDS-PAGE), staining with antibodies, fluorescent activated cell sorter (FACS), pyrolysis mass spectrometry, Fourier-Transform Infrared Spectrometry, Raman spectrometry, GC-MS, and LC-Electrospray and cap-LC-tandem-electrospray mass spectrometries, and the like. Novel bioactivities can also be screened using methods, or variations thereof, described in U.S. Patent No. 6,057,103. Furthermore, one or more, or, all the polypeptides of a cell can be measured using a protein array.

Methods of Using DAT or Oxidoreductase Nucleic Acids and Polypeptides

The AT or oxidoreductase polypeptides or the AT or oxidoreductase nucleic acids encoding such AT and oxidoreductase polypeptides, respectively, can be used to facilitate the conversion of tryptophan to indole-3-pyruvate and/or to facilitate the conversion of MP to monatin (or the reverse reaction). It is noted that the reactions described herein are not limited to any particular method, unless otherwise stated. The reactions disclosed herein can take place, for example, *in vivo*, *in vitro*, or a combination thereof.

Constructs containing AT or oxidoreductase nucleic acid molecules are provided. Constructs, including expression vectors, suitable for expressing an AT or oxidoreductase polypeptide are commercially available and/or readily produced by recombinant DNA technology methods routine in the art. Representative constructs or vectors include, without limitation, replicons (e.g., RNA replicons, bacteriophages), autonomous self-replicating circular or linear DNA or RNA, a viral vector (e.g., an adenovirus vector, a retroviral vector or an adeno-associated viral vector), a plasmid, a phage, a phagemid, a cosmid, a fosmid, a bacteriophage or an artificial chromosome. The cloning vehicle can comprise an artificial chromosome comprising a bacterial artificial chromosome (BAC), a bacteriophage P1-derived vector (PAC), a yeast artificial chromosome (YAC), or a mammalian artificial chromosome (MAC). Exemplary vectors include, without limitation, pBR322 (ATCC 37017), pKK223-3, pSVK3, pBPV, pMSG, and pSVL (Pharmacia Fine Chemicals, Uppsala, Sweden), GEM1 (Promega Biotec, Madison, WI, USA) pQE70, pQE60, pQE-9 (Qiagen), pD10, psiX174 pBluescript II KS, pNH8A, pNH16a, pNH18A, pNH46A, pSV2CAT, pOG44, pXT1, pSG (Stratagene), ptrc99a, pKK223-3,

pKK233-3, DR540, pRIT5 (Pharmacia), pKK232-8 and pCM7. See, also, U.S. Patent No. 5,217,879 for a description of representative plasmids, viruses, and the like.

A vector or construct containing an AT or oxidoreductase nucleic acid molecule can have elements necessary for expression operably linked to the AT or oxidoreductase nucleic acid. Elements necessary for expression include nucleic acid sequences that direct and regulate expression of nucleic acid coding sequences. One example of an element necessary for expression is a promoter sequence. Promoter sequences are sequences that are capable of driving transcription of a coding sequence. A promoter sequence can be, for example, an AT or oxidoreductase promoter sequence, or a non-AT or non-oxidoreductase promoter sequence. Non-AT and non-oxidoreductase promoters include, for example, bacterial promoters such as lacI, lacZ, T3, T7, gpt, lambda PR, lambdaPL and trp as well as eukaryotic promoters such as CMV immediate early, HSV thymidine kinase, early and late SV40, LTRs from retrovirus, and mouse metallothionein I. Promoters also can be, for example, constitutive, inducible, and/or tissue-specific. A representative constitutive promoter is the CaMV 35S; representative inducible promoters include, for example, arabinose, tetracycline-inducible and salicylic acid-responsive promoters.

Additional elements necessary for expression can include introns, enhancer sequences (e.g., an SV40 enhancer), response elements, or inducible elements that modulate expression of a nucleic acid. Elements necessary for expression can include a leader or signal sequence. See, for example, SEQ ID NO:156, which is a DAT polypeptide having a leader sequence. Elements necessary for expression also can include, for example, a ribosome binding site for translation initiation, splice donor and acceptor sites, and a transcription terminator. Elements necessary for expression can be of bacterial, yeast, insect, mammalian, or viral origin, and vectors or constructs can contain a combination of elements from different origins. Elements necessary for expression are described, for example, in Goeddel, 1990, *Gene Expression Technology: Methods in Enzymology*, 185, Academic Press, San Diego, CA.

A vector or construct as described herein further can include sequences such as those encoding a selectable marker (e.g., genes encoding dihydrofolate reductase or genes conferring neomycin resistance for eukaryotic cells; genes conferring tetracycline or ampicillin resistance for *E. coli*; and the gene encoding TRP1 for *S. cerevisiae*), sequences that can be used in purification of an AT or oxidoreductase polypeptide (e.g., 6xHis tag), and one or more sequences involved in replication of the vector or construct (e.g., origins of replication). In addition, a vector or construct can contain, for example, one or two regions that have sequence homology for integrating the vector or construct. Vectors and constructs for genomic integration are well known in the art.

As used herein, operably linked means that a promoter and/or other regulatory element(s) are positioned in a vector or construct relative to an AT or oxidoreductase nucleic acid in such a way as to direct or regulate expression of the AT or oxidoreductase nucleic acid. Generally, promoter and other elements necessary for expression that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are cis-acting. Some transcriptional regulatory sequences such as enhancers, however, need not be physically contiguous or located in close proximity to the coding sequences whose expression they enhance.

Also provided are host cells. Host cells generally contain a nucleic acid sequence of the invention, e.g., a sequence encoding an AT or an oxidoreductase, or a vector or construct as described herein. The host cell may be any of the host cells familiar to those skilled in the art such as prokaryotic cells or eukaryotic cells including bacterial cells, fungal cells, yeast cells, mammalian cells, insect cells, or plant cells. Exemplary bacterial cells include any species within the genera *Escherichia*, *Bacillus*, *Streptomyces*, *Salmonella*, *Pseudomonas* and *Staphylococcus*, including, e.g., *E. coli*, *L. lactis*, *B. subtilis*, *B. cereus*, *S. typhimurium*, *P. fluorescens*. Exemplary fungal cells include any species of *Aspergillus*, and exemplary yeast cells include any species of *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Schwanniomyces*, including *P. pastoris*, *S. cerevisiae*, or *S. pombe*. Exemplary insect cells include any species of *Spodoptera* or *Drosophila*, including *Drosophila S2* and *Spodoptera Sf9*. Exemplary animal cells include CHO, COS, Bowes melanoma, C127, 3T3, HeLa and BHK cell lines. See, for example, Gluzman, 1981, *Cell*, 23:175. The selection of an appropriate host is within the abilities of those skilled in the art.

Techniques for introducing nucleic acid into a wide variety of cells are well known and described in the technical and scientific literature. A vector or construct can be introduced into host cells using any of a variety of techniques, including transformation, transfection, transduction, viral infection, gene guns, or Ti-mediated gene transfer. Particular methods include calcium phosphate transfection, DEAE-Dextran mediated transfection, lipofection, or electroporation (Davis et al., 1986, *Basic Methods in Molecular Biology*). Exemplary methods include $CaPO_4$ precipitation, liposome fusion, lipofection (e.g., LIPOFECTIN™), electroporation, viral infection, etc. The AT or oxidoreductase nucleic acids may stably integrate into the genome of the host cell (for example, with retroviral introduction) or may exist either transiently or stably in the cytoplasm (i.e. through the use of traditional plasmids, utilizing standard regulatory sequences, selection markers, etc.).

The content of host cells usually is harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells

employed for expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or the use of cell lysing agents. Such methods are well known to those skilled in the art. The expressed polypeptide or fragment thereof can be recovered and purified from cell cultures by methods including, but not limited to, precipitation (e.g., ammonium sulfate or ethanol), acid extraction, chromatography (e.g., anion or cation exchange, phosphocellulose, hydrophobic interaction, affinity, hydroxylapatite and lectin). If desired, high performance liquid chromatography (HPLC) can be employed for final purification steps.

Cell-free translation systems can also be employed to produce a polypeptide of the invention. Cell-free translation systems can use mRNAs transcribed from a DNA construct comprising a promoter operably linked to a nucleic acid encoding the polypeptide or fragment thereof. In some aspects, the DNA construct may be linearized prior to conducting an in vitro transcription reaction. The transcribed mRNA is then incubated with an appropriate cell-free translation extract, such as a rabbit reticulocyte extract, to produce the desired polypeptide or fragment thereof.

An AT or oxidoreductase polypeptide, a fragment, or a variant thereof can be assayed for activity by any number of methods. Methods of detecting or measuring the activity of an enzymatic polypeptide generally include combining a polypeptide, fragment or variant thereof with an appropriate substrate and determining whether the amount of substrate decreases and/or the amount of product or by-product increases. The substrates used to evaluate the activity of DATs disclosed herein typically were tryptophan and/or R-MP, and the products were indole-3-pyruvate and/or R,R-monatin. In addition to a tryptophan or MP substrate, it is expected that polypeptides disclosed herein also will utilize substituted tryptophan and/or MP substrates such as, without limitation, chlorinated tryptophan or 5-hydroxytryptophan. In addition, a by-product of the conversion of MP to monatin (e.g., 4-hydroxy-4-methyl glutamic acid (HMG)) can be monitored or measured.

Methods for evaluating AT activity are described, for example, in Sugio et al., 1995, *Biochemistry*, 34:9661-9669; Ro et al., 1996, *FEBS Lett.*, 398:141-145; or Gutierrez et al., 2000, *Eur. J. Biochem.*, 267, 7218-7223. In addition, methods for evaluating dehydrogenase activity are described, for example, in Lee et al., 2006, *AEM*, 72(2):1588-1594; and Mayer, 2002, *J. Biomolecular Screening*, 7(2):135-140. In addition, methods of evaluating candidate polypeptides for DAT activity are described in Part A and Part B of the Example section herein. For the purposes of determining whether or not a polypeptide falls within the scope of the invention, the methods described in Part B of the Example section are employed.

Typically, an AT or oxidoreductase polypeptide exhibits activity in the range of between about 0.05 to 20 units (e.g., about 0.05, 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, 0.80, 0.90, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 19.5 or more units). As used herein, a unit equals one μmol of product released per minute per mg of enzyme. In one embodiment, one unit of activity for an AT polypeptide is one μmol of alpha-keto acid or ketone produced per minute per mg of enzyme (formed from the respective alpha-amino acid or amine). In an alternative embodiment, one unit of activity for an aminotransferase polypeptide is one μmol of alpha-amino acid or amine produced per minute per mg of enzyme (formed from the respective alpha-keto acid or ketone).

The conversion of tryptophan to indole-3-pyruvate or the conversion of MP to monatin using one or more of the AT or oxidoreductase nucleic acids or polypeptides disclosed herein can be performed *in vitro* or *in vivo*, in solution or in a host cell, in series or in parallel. When one or more reactions are performed *in vitro*, the desired ingredients for the reaction(s) can be combined by admixture in an aqueous reaction medium or solution and maintained for a period of time sufficient for the desired product(s) to be produced. Alternatively, one or more AT or oxidoreductase polypeptides used in the one or more of the reactions described herein can be immobilized onto a solid support. Examples of solid supports include those that contain epoxy, aldehyde, chelators, or primary amine groups. Specific examples of suitable solid supports include, but are not limited to, Eupergit® C (Rohm and Haas Company, Philadelphia, PA) resin beads and SEPABEADS® EC-EP (Resindion).

To generate indole-3-pyruvate from typtophan or monatin from MP *in vivo*, an AT or oxidoreductase nucleic acid (e.g., an expression vector) can be introduced into any of the host cells described herein. Depending upon the host cell, many or all of the co-factors (e.g., a metal ion, a co-enzyme, a pyridoxal-phosphate, or a phosphopanthetheine) and/or substrates necessary for the conversion reactions to take place can be provided in the culture medium. After allowing the *in vitro* or *in vivo* reaction to proceed, the efficiency of the conversion can be evaluated by determining whether the amount of substrate has decreased or the amount of product has increased.

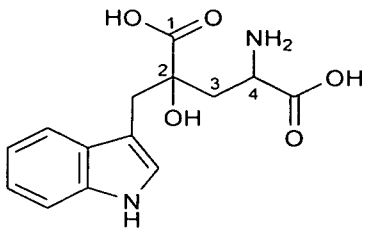
In some embodiments, the activity of one or more of the AT or oxidoreductase polypeptides disclosed herein can be improved or optimized using any number of strategies known to those of skill in the art. For example, the *in vivo* or *in vitro* conditions under which one or more reactions are performed such as pH or temperature can be adjusted to improve or optimize the activity of a polypeptide. In addition, the activity of a polypeptide can be improved or optimized by re-cloning the AT or oxidoreductase nucleic acid into a different vector or construct and/or by using a different host cell. For example, a host cell can be used that has been

genetically engineered or selected to exhibit increased uptake or production of tryptophan (see, for example, U.S. Patent No. 5,728,555). Further, the activity of an AT or oxidoreductase polypeptide can be improved or optimized by ensuring or assisting in the proper folding of the polypeptide (e.g., by using chaperone polypeptides) or in the proper post-translational modifications such as, but not limited to, acetylation, acylation, ADP-ribosylation, amidation, glycosylation, hydroxylation, iodination, methylation, myristolyation, oxidation, pegylation, phosphorylation, prenylation, , selenoylation, sulfation, disulfide bond formation, and demethylation as well as covalent attachment of molecules such as flavin, a heme moiety, a nucleotide or nucleotide derivative, a lipid or lipid derivative, and/or a phosphytidylinositol. In addition, the solubility of a polypeptide can be increased using any number of methods known in the art such as, but not limited to, low temperature expression or periplasmic expression.

A number of polypeptides were identified herein that exhibit DAT activity using tryptophan and/or MP as a substrate. Specifically, SEQ ID NO:950, 946, 948, 892, 894, 866, 870, 870 T242N, 872, 874, 878, 880, 882, 884, 902, 910, 918, 176, 178, 154, 220, 156, 216, 238, 224, 230, 232, 214, *Cb*DAT, *Ca*DAT and *Ls*DAT exhibit DAT activity. Notably, SEQ ID NO:946, 892, 894, 220, 176, 1064, 1066 and 1068 exhibited very high activity under the conditions described in Part B of the Examples. It is noted that SEQ ID NO:220 and 894 produced low levels of the HMG by-product during the conversion of MP to monatin.

Use of Aminotransferase or Oxidoreductase Nucleic Acids or Polypeptides in the Production of Monatin

One or more of the DAT polypeptides disclosed herein can be used in the production of monatin. Monatin is a high-intensity sweetener having the chemical formula:



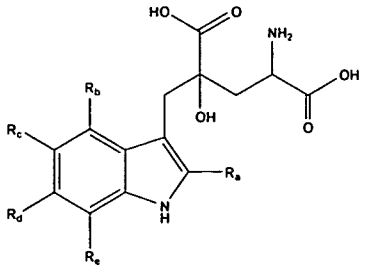
Monatin includes two chiral centers leading to four potential stereoisomeric configurations. The R,R configuration (the "R,R stereoisomer" or "R,R monatin"); the S,S configuration (the "S,S stereoisomer" or "S,S monatin"); the R,S configuration (the "R,S stereoisomer" or "R,S monatin"); and the S,R configuration (the "S,R stereoisomer" or "S,R monatin"). As used herein, unless stated otherwise, the term "monatin" is used to refer to compositions including all four stereoisomers of monatin, compositions including any

combination of monatin stereoisomers, (e.g., a composition including only the R,R and S,S, stereoisomers of monatin), as well as a single isomeric form (or any of the salts thereof). Due to various numbering systems for monatin, monatin is known by a number of alternative chemical names, including: 2-hydroxy-2-(indol-3-ylmethyl)-4-aminoglutaric acid; 4-amino-2-hydroxy-2-(1H-indol-3-ylmethyl)-pentanedioic acid; 4-hydroxy-4-(3-indolylmethyl)glutamic acid; and, 3-(1-amino-1,3-dicarboxy-3-hydroxy-but-4-yl)indole.

Methods of producing various stereoisomers of monatin (e.g., R,R monatin) are disclosed in, for example, WO 07/133183 and WO 07/103389. One or more of the DAT polypeptides disclosed herein, in the presence of tryptophan, can be used in methods known to those of skill in the art to make a monatin composition. As disclosed in both WO 07/133183 and WO 07/103389, the conversion of indole-3-pyruvate (or derivatives thereof; see, for example, WO 07/103389) to 2-hydroxy 2-(indol-3ylmethyl)-4-keto glutaric acid ("monatin precursor" or "MP") dictates the first chiral center of monatin, while the conversion of MP to monatin dictates the second chiral center. In on embodiment, one or more of the conversions required to produce monatin is catalyzed by more than one enzyme, for example, a mixture of enzymes, so that the resulting composition or preparation contains a desired percentage (e.g., minimum and/or maximum) of one or more of the monatin stereoisomers (e.g., R,R monatin). Alternatively, monatin made by two separate engineered pathways according to the methods of the invention be combined to produce a composition or preparation containing such desired percentage of each monatin stereoisomer(s).

Monatin that is produced utilizing one or more of the AT polypeptides disclosed herein can be at least about 0.5-30% R,R-monatin by weight of the total monatin produced. In other embodiments, the monatin produced using one or more of the polypeptides or biosynthetic pathways disclosed herein, is greater than 30% R,R-monatin, by weight of the total monatin produced; for example, the R,R-monatin is 40%, 50%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or 99% of the total monatin produced. Alternatively, various amounts of two or more preparations of monatin can be combined so as to result in a preparation that is a desired percentage of R,R-monatin. For example, a monatin preparation that is 30% R,R-monatin can be combined with a monatin preparation that is 90% R,R-monatin; if equal amounts of 30% and 90% R,R-monatin preparations are combined, the resulting monatin preparation would be 60% R,R-monatin.

Monatin produced using one or more of the DAT polypeptides disclosed herein can be for example, a derivative. "Monatin derivatives" have the following structure:



wherein, R_a, R_b, R_c, R_d, and R_e each independently represent any substituent selected from a hydrogen atom, a hydroxyl group, a C_1-C_3 alkyl group, a C_1-C_3 alkoxy group, an amino group, or a halogen atom, such as an iodine atom, bromine atom, chlorine atom, or fluorine atom. However, R_a, R_b, R_c, R_d, and R_e cannot simultaneously all be hydrogen. Alternatively, R_b and R_c, and/or R_d and R_e may together form a C_1-C_4 alkylene group, respectively. "Substituted monatin" refers to, for example, halogenated or chlorinated monatin or monatin derivatives. See, for example, U.S. Publication No. 2005/0118317.

Monatin derivatives also can be used as sweeteners. For example, chlorinated D-tryptophan, particularly 6-chloro-D-tryptophan, which has structural similarities to R,R monatin, has been identified as a non-nutritive sweetener. Similarly, halogenated and hydroxy-substituted forms of monatin have been found to be sweet. See, for example, U.S. Publication No. 2005/0118317. Substituted indoles have been shown in the literature to be suitable substrates for PLP-enzymes and have yielded substituted tryptophans. See, for example, Fukuda et al.,1971, *Appl. Environ. Microbiol.*, 21:841-43. The halogen does not appear to sterically hinder the catalytic mechanism or the enantiospecificity of the enzyme. Therefore, halogens and hydroxyl groups should be substitutable for hydrogen, particularly on positions 1-4 of the benzene ring in the indole of tryptophan, without interfering in subsequent conversions to D- or L-tryptophan, indole-3-pyruvate, MP, or monatin.

One or more of the DAT polypeptides disclosed herein, with or without one or more additional polypeptides, can be used in the production of monatin. A DAT polypeptide (or nucleic acid molecule encoding such a DAT polypeptide) can be used in the conversion of tryptophan to indole-3-pyruvic acid (in the presence of an amino acceptor) and in the conversion of MP to monatin. The intermediate step between those two reactions is the conversion of indole-3-pyruvic acid to MP, which requires the presence of a C3 carbon source such as pyruvate, oxaloacetate or serine. The use of a DAT polypeptide in the conversion step from MP to monatin results in the R configuration at the second chiral center. It is noted that SEQ ID NO:946, 950, 220 and 948 produced high amounts of R,R monatin.

The conversion of indole-3-pyruvate (or indole-3-pyruvic acid) to MP can occur in the absence of an enzyme (i.e., an aldol condensation), but also can be facilitated by a polypeptide. The chirality at the first chiral center is determined by the enantiospecificity of the reaction converting indole-3-pyruvate to MP. If the MP formation reaction is not facilitated by an enzyme, a racemic mixture of R-MP and S-MP is typically formed in the absence of a chiral auxiliary. Enzymes that facilitate the conversion of indole-3-pyruvate to MP include, for example, a synthase / lyase (EC 4.1.3.- and 4.1.2.-), specifically those in classes EC 4.1.3.16 and EC 4.1.3.17. These classes include carbon-carbon synthases/lyases, such as aldolases that catalyze the condensation of two carboxylic acid substrates. Enzyme class EC 4.1.3.- are those synthases/lyases that form carbon-carbon bonds utilizing oxo-acid substrates (such as indole-3-pyruvate) as the electrophile, while EC 4.1.2.- are synthases/lyases that form carbon-carbon bonds utilizing aldehyde substrates (such as benzaldehyde) as the electrophile. For example, KHG aldolase (EC 4.1.3.16) and HMG aldolase (EC 4.1.3.17), are known to convert indole-3-pyruvate and pyruvate to MP. Herein, the term HMG aldolase is used to mean any polypeptide with 4-hydroxy-4-methyl-2-oxoglutarate aldolase activity. Suitable examples of HMG aldolases include *Comamonas testosteroni* ProA and *Sinorhizobium meliloti* ProA (NCBI Accession No. CAC46344).

When one or more of the conversion reactions in the pathway to producing monatin are to be performed *in vivo*, a person of ordinary skill in the art may optimize production of monatin in a microorganism, including R,R monatin, by various routine methods. Such a microorganism can be one that naturally is better than other microorganisms in one or more of the following characteristics, or that has been modified to exhibit one or more of the following characterisitics, which typically result in improved production of monatin (relative to the microorganism before such modification). Such characteristics include, without limitation, an increase in the ability of a microorganism to take-up tryptophan (e.g., D-tryptophan); an increase in the ability of a microorganism to take-up indole-3-pyruvate; a decrease in the ability of a microorganism to secrete indole-3-pyruvate; a decrease in the amount of degradation of indole-3-pyruvate in the microorganism; and/or a decrease in the toxicity of D-tryptophan to a microorganism. Such characteristics and strategies for obtaining microorganisms exhibiting one or more such characteristics are described in, for example, WO 07/133183 and WO 07/103389.

Monatin, or an intermediate of the tryptophan to monatin biosynthetic pathway (including indole-3-pyruvate and MP) produced using one or more of the AT or oxidoreductase polypeptides disclosed herein can be purified from the components of the reaction. In one embodiment, the monatin or an intermediate can be purified simply by removing the substance that is to be purified from the enzyme preparation in which it was synthesized. In other

embodiments, monatin or an intermediate is purified from a preparation in which it was synthesized so that the resulting "purified" composition or preparation is at least about 5-60% monatin by weight of total organic compounds. In another embodiment, the monatin or an intermediate can be purified to a degree of purity of at least about 70%, 80%, 90%, 95% or 99% by weight of total organic compounds. The monatin produced using one or more of the polypeptides or biosynthetic pathways disclosed herein can be purified from the components of the reaction by any method known to a person of ordinary skill in the art (e.g., repeatedly recrystallization).

In accordance with the present invention, there may be employed conventional molecular biology, microbiology, biochemical, and chemical techniques within the skill of the art. Such techniques are explained fully in the literature. The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

The aminotransferases and oxidoreductases described herein were obtained using a selection strategy. In this selection strategy, environmental DNA libraries were constructed in a bacterial host strain that exhibits L-tryptophan auxotrophy. Library clones were innoculated onto media containing D-tryptophan (but lacking L-tryptophan). The only clones that could grow are those that expressed a gene on one of the discrete environmental DNA fragments that encoded an enzyme active on D-tryptophan. For example, clones were identified that expressed an active tryptophan racemase and were able to convert D-tryptophan to L-tryptophan. Additionally, clones were identified that expressed an oxidoreductase (such as an amino acid oxidase or a dehydrogenase) that could convert D-tryptophan to an intermediate that the host cell could, in turn, convert to L-tryptophan. In the case of oxidoreductases such as aminoacid oxidases and dehydrogenases, one such intermediate is indole-3-pyruvate.

The Examples in Part A describe the methodologies used for initial characterization of the candidate DAT and oxidoreductase nucleic acids and the encoded polypeptides. Further characterization of particular nucleic acids and polypeptides is described in Part B.

Part A

Example 1—Growth and Assay Procedures #1

Enzyme Preparation

Glycerol stocks were used to inoculate flasks containing 50 mL of LB medium with the appropriate antibiotic. The starter cultures were grown overnight at 37°C with shaking at 230

rpm and the OD_{600nm} was checked. The starter culture was used to inoculate 400 mL to OD_{600nm} of 0.05. The culture was incubated at 37°C with shaking at 230 rpm.

Cultures were induced with 1 mM IPTG when the OD_{600nm} was between 0.5 and 0.8 and incubated at 30°C and 230 rpm overnight. Cultures were harvested by pelleting cells by centrifugation at 4000 rpm for 15 minutes. The supernatant was poured off and the pellet was either frozen for later use or resuspended in 20 mL of 50 mM sodium phosphate buffer (pH 7.5) supplemented with 26 U/mL of DNAse and lysed using a microfluidizer (Microfluidics Corporation, Newton, MA) per the manufacturer's instructions. The clarified lysate was collected and centrifuged at 11,000 rpm for 30 minutes. The supernatant was collected in a clean tube and filtered through a 0.2 μm filter. Five mL aliquots of the clarified lysate were placed in a vial and freeze-dried using a lyophilizer (Virtis Company, Gardinier, NY) per the manufacturer's instructions. Approximately 1 mL of the clarified lysate was retained for protein quantitation using the Bio-Rad Protein Assay Reagent (Bio-Rad, Hercules, CA) and SDS-PAGE analysis. Then, the amount of protein in each lyophilized sample was calculated.

Activity assay

Enzymes for activity assays were prepared in 50 mM sodium phosphate pH 7.5. DAT assays were usually performed using approximately 1 mg/mL total protein.

DAT assay using RR-monatin substrate

Twenty-five mM RR-monatin, 25 mM pyruvic acid sodium salt, 0.08 mM PLP, 90 mM sodium phosphate pH 8.0 and 0.8 mg/mL DAT (total protein) prepared as described above (under 'Enzyme Preparation' section) were combined and incubated at 30°C at 300 rpm. At various timepoints (generally 0, 2, 4, and 24 hours), 50 μL of the reaction product was transferred to 150 μL of ice cold acetonitrile, and the sample vortexed for 30 seconds. Samples were centrifuged at 13,200 rpm for 10 minutes at 4°C, and the supernatant was passed through a 0.45 μm filter. The filtrate was diluted 10-fold in methanol, and samples were analyzed by LC/MS/MS to monitor the D-alanine formed (described in this Example below under 'LC/MS/MS method for detecting D-alanine or R,R-monatin' section).

DAT assay using tryptophan substrate

Ten mM D-tryptophan, 25 mM pyruvic acid sodium salt, 0.08 mM PLP, 90 mM sodium phosphate pH 8.0, and 0.8 mg/mL DAT (total protein) prepared as described above (under 'Enzyme Preparation' section) were combined and incubated at 30°C and 300 rpm. At timepoints (generally 0, 2, 4, and 24 hours), 50 μL of the reaction product was transferred to 150 μL of ice cold acetonitrile, vortexed for 30 seconds, and centrifuged at 13,200 rpm for 10 minutes at 4°C. The supernatant was passed through a 0.45 μm filter and the filtrate was diluted 10-fold in methanol. Samples were analyzed by LC/MS/MS to monitor the D-alanine formed

(described in this Example below under 'LC/MS/MS method for detecting D-alanine or R,R-monatin' section).

LC/MS/MS method for detecting D-alanine or R,R-monatin

LC/MS/MS screening was achieved by injecting samples from 96-well plates using a CTCPal auto-sampler (LEAP Technologies, Carrboro, NC) into a 30/70 H_2O/Acn (0.1% formic acid) mixture provided by LC-10ADvp pumps (Shimadzu, Kyoto, Japan) at 1.0 mL/min through a Zorbax Eclipse XDB-C8 (2.1 x 50 mm) column and into the API4000 TurboIon-Spray triple-quad mass spectrometer (Applied Biosystems, Foster City, CA).

Ion spray and Multiple Reaction Monitoring (MRM) were performed for the analytes of interest in the positive ion mode. alanine: parent/daughter ions: 90.12/44.25 monatin: parent/daughter ions: 293.11/130.15.

Example 2—Activity of DATs Using Assay Procedures #1

The vector pSE420-cHis is a derivative of pSE420 (Invitrogen, Carlsbad, CA). For pSE420-cHis, the vector was cut with *Nco*I and *Hind* III, and ligated with C-His. C-His: 5'-CCA TGG GAG GAT CCA GAT CTC ATC ACC ATC ACC ATC ACT AAG CTT (SEQ ID NO:977). The expression of the His-tag in this vector depends on the choice of host and stop codon. That is, if a TAG stop codon and a supE host are used, the His-tag is expressed; if a TAG stop codon and a non supE host are used, the His-tag is not expressed. Unless indicated otherwise, the His-tag was not expressed in these experiments.

The DAT subclones were in the pSE420-cHis vector / *E.coli* HMS174 host (Novagen, San Diego, CA) with the exception of the following subclones: SEQ ID NO:930, 932, 936 were in the pET101 D-Topo vector/ BL21Star(DE3) host (Invitrogen, Carlsbad, CA); SEQ ID NO:934 was in the pET101 D-Topo vector/ BL21 Codon PlusRIL host (Stratagene, La Jolla, CA); SEQ ID NO:938, 942, 944, 946 were in the pSE420 vector / XL1Blue host (Stratagene, La Jolla, CA); SEQ ID NO:940, 948, 950, 962 and 966 were in the pSE420-c-His vector / XL1Blue host (Stratagene, La Jolla, CA); and SEQ ID NO:928 was in the pQET1 vector / M15pREP4 host (pQET1 described in U.S. Patent Nos. 5,814,473 and 6,074,860; M15pREP4 from Qiagen; Valencia, CA).

The subclones were grown, lysed and lyophilized according to the procedures described in Example 1. Samples were tested for activity on R,R-monatin as well as D-tryptophan (as described in Example 1). For the monatin DAT assay, DATs were incubated with 25 mM R,R-monatin, 25 mM pyruvic acid sodium salt, and 0.08 mM PLP (pH 8) at 30°C. For the D-tryptophan DAT assay, DATs were incubated with 10 mM D-tryptophan, 25 mM pyruvic acid

soldium salt, and 0.08 mM PLP (pH 8) at 30°C. All DATs were loaded at 0.8 mg/mL total protein in both assays.

At indicated timepoints, 50 μL of the reaction product was added to 150 μL of ice-cold acetonitrile. Samples were vortexed for 30 seconds and the supernatant was then diluted ten-fold in methanol. Samples were then analyzed by LC/MS/MS (as described in Example 1) to monitor the D-alanine formed. The tables below show the D-aminotransferase activity on both substrates.

Table 1: Activity of D-aminotransferase subclones on R,R-monatin and D-tryptophan

SEQ ID NO:	Activity on R,R-monatin	Activity on D-tryptophan	Relative Expression
	μg/mL D-alanine formed at indicated hour	μg/mL D-alanine formed at indicated hour	
928	30@24 hr	NT	+
938	122@24 hr	NT	++
940	5@24 hr	NT	ND
942	12@24 hr	NT	ND
944	75@24 hr	NT	ND
946	39@24 hr	NT	ND
948	200@0.5 hr	441@0.5 hr	ND
950	75@0.5 hr	452@0.5 hr	ND
962	NT	NT	+
964	7@24 hr	ND@24 hr	++
966	NT	NT	++
968	6.7@24 hr	52@24 hr	+++
886 (expressed in XL1Blue cells)	NT	NT	++
886 (expressed in *E. coli* HMS174 cells)	15.4@24 hr	143@24 hr	+++

NT, not tested; ND, not detected under conditions used; +, low expression, ++, moderate expression, +++, high expression

Table 1: Activity of D-aminotransferase subclones on R,R-monatin and D-tryptophan (con't)

Subclone name	Activity on RR-monatin	Activity on D-tryptophan	Relative expression
	μg/mL D-alanine formed at indicated hour	μg/mL D-alanine formed at indicated hour	
888 (expressed in XL1 Blue cells)	NT	NT	++
888 (expressed in HMS174 cells)	7@24 hr	317@24 hr	+++
890 (expressed in XL1 Blue cells)	NT	NT	++
890 (expressed in HMS174 cells)	54@24 hr	278@24 hr	+++
892 (expressed in XL1 Blue cells)	NT	NT	+

892 (expressed in HMS174 cells)	113@24 hr	<5@24 hr	++
894 (expressed in XL1 Blue cells)	NT	NT	ND
894 (expressed in HMS174 cells)	16@24 hr	116@24 hr	+
866	28@24 hr	NT	+++
868	<1@24 hr	NT	+++
970	10.8@24 hr	NT	+
870	123.5@24 hr	NT	+++
872	62.3@24 hr	NT	+++
874	46.5@24 hr	NT	+++
876	44@24 hr	NT	++
878	37@24 hr	NT	+++
972	<5@24 hr	NT	+
880	72.4@24 hr	79.6@24 hr	+
882	158.8@24 hr	344@2 hr	+++
884	290@24 hr	363@2 hr	++
896	54@24 hr	450@2 hr	+++
898	466@24 hr	300@24 hr	+
900	135@24 hr	154@24 hr	+
902	280@24 hr	130@24 hr	++
904	170@24 hr	140@24 hr	+
906	700@24 hr	500@24 hr	+++
908	55@24 hr	45@24 hr	+ insoluble

NT, not tested; ND, not detected; +, low expression; ++, moderate expression; +++, high expression

Table 1. Activity of D-aminotransferase subclones on R,R-monatin and D-tryptophan (con't)

Subclone name	Activity on RR-monatin	Activity on D-tryptophan	Relative Expression
	μg/mL D-alanine formed at indicated hour	μg/mL D-alanine formed at indicated hour	
910	384@24 hr	240@24 hr	+++
912	NT	NT	ND
914	NT	NT	ND
916	NT	NT	ND
918	NT	NT	ND
920	NT	NT	ND
922	NT	NT	ND
924	NT	NT	ND
926	NT	NT	ND
974 (expressed in HMS174 cells)	NT	NT	ND
974 (expressed in XL1 Blue cells)	NT	NT	ND
930	NT	NT	ND
932	NT	NT	ND
934	NT	NT	ND
936	NT	NT	ND

976 (expressed in LX1 Blue cells)	NT	<5@24 hr	++

NT, not tested; ND, not detected; +, low expression; ++, moderate expression; +++, high expression

It should be noted that there are only very conservative differences between the subclones listed above and their native sequences that are also in the sequence listing. For example, quite often, a start or stop codon was modified to be more efficient for expression in *E. coli*. It is expected that cloning of the wildtype sequences would give similar results in terms of DAT activity. For clarification purposes, the following table shows the relationship between a number of the clones and subclones described herein.

Clone/subclone pair	SEQ ID NO:	Activity	Sequence type (clone or subclone)
1	31, 32	D-AT	Clone
1	867, 868	D-AT	Subclone
2	955, 956	D-AT	Clone
2	929, 930	D-AT	Subclone
3	957, 958	D-AT	Clone
3	931, 932	D-AT	Subclone
4	959, 960	D-AT	Clone
4	935, 936	D-AT	Subclone
5	41, 42	D-AT	Clone
5	869, 870	D-AT	Subclone
6	7, 8	D-AT	Clone
6	943, 944	D-AT	Subclone
7	11, 12	D-AT	Clone
7	941, 942	D-AT	Subclone
8	83, 84	D-AT	Clone
8	879, 880	D-AT	Subclone
9	151, 152	D-AT	Clone
9	913, 914	D-AT	Subclone
10	951, 952	D-AT	Clone
10	933, 934	D-AT	Subclone
11	75, 76	D-AT	Clone
11	881, 882	D-AT	Subclone
12	87, 88	D-AT	Clone
12	883, 884	D-AT	Subclone
13	163, 164	D-AT	Clone
13	921, 922	D-AT	Subclone
14	145, 146	D-AT	Clone
14	919, 920	D-AT	Subclone
15	149, 150	D-AT	Clone
15	925, 926	D-AT	Subclone
16	147, 148	D-AT	Clone
16	915, 916	D-AT	Subclone

17	15, 16	D-AT	Clone
17	947, 948	D-AT	Subclone
18	17, 18	D-AT	Clone
18	949, 950	D-AT	Subclone
19	3, 4	D-AT	Clone
19	937, 938	D-AT	Subclone
20	5, 6	D-AT	Clone
20	939, 940	D-AT	Subclone
21	161, 162	D-AT	Clone
21	923, 924	D-AT	Subclone
22	953, 954	D-AT	Clone
22	927, 928	D-AT	Subclone
23	19, 20	D-AT	Clone
23	885, 886	D-AT	Subclone
24	21, 22	D-AT	Clone
24	891, 892	D-AT	Subclone
25	23, 24	D-AT	Clone
25	893, 894	D-AT	Subclone
26	13, 14	D-AT	Clone
26	945, 946	D-AT	Subclone
27	143, 144	D-AT	Clone
27	917, 918	D-AT	Subclone
28	43, 44	D-AT	Clone
28	871, 872	D-AT	Subclone
29	45, 46	D-AT	Clone
29	873, 874	D-AT	Subclone
30	49, 50	D-AT	Clone
30	897, 898	D-AT	Subclone
31	51, 52	D-AT	Clone
31	875, 876	D-AT	Subclone
32	37, 38	D-AT	Clone
32	877, 878	D-AT	Subclone
33	25, 26	D-AT	Clone
33	889, 890	D-AT	Subclone
34	27, 28	D-AT	Clone
34	887, 888	D-AT	Subclone
35	131, 132	D-AT	Clone
35	909, 910	D-AT	Subclone
36	53, 54	D-AT	Clone
36	865, 866	D-AT	Subclone
37	29, 30	D-AT	Clone
37	895, 896	D-AT	Subclone
38	125, 126	D-AT	Clone
38	907, 908	D-AT	Subclone
39	133, 134	D-AT	Clone
39	911, 912	D-AT	Subclone
40	127, 128	D-AT	Clone
40	899, 900	D-AT	Subclone
41	137, 138	D-AT	Clone
41	901, 902	D-AT	Subclone
42	139, 140	D-AT	Clone

42	903, 904	D-AT	Subclone
43	129, 130	D-AT	Clone
43	905, 906	D-AT	Subclone
44	33, 34	D-AT	Clone
44	969, 970	D-AT	Subclone
45	219, 220	D-AT	Clone
45	973, 974	D-AT	Subclone
46	39, 40	D-AT	Clone
46	971, 972	D-AT	Subclone
47	1, 2	D-AT	Clone
47	975, 976	D-AT	Subclone
48	253, 254	Dehydrogenase	Clone
48	961, 962	Dehydrogenase	Subclone

Part B

Example 3—Detection of Monatin, MP, Tryptophan, Alanine, and HMG

This example describes the analytical methodology associated with the further characterization of selected D-aminotransferase (DAT) enzymes.

LC/MS/MS Multiple Reaction Monitoring (MRM) Analysis of monatin and tryptophan

Analyses of mixtures for monatin and tryptophan derived from biochemical reactions were performed using a Waters/Micromass® liquid chromatography-tandem mass spectrometry (LC/MS/MS) instrument including a Waters 2795 liquid chromatograph with a Waters 996 Photo-Diode Array (PDA) absorbance monitor placed in series between the chromatograph and a Micromass® Quattro Ultima® triple quadrupole mass spectrometer. LC separations were made using an Xterra MS C8 reversed-phase chromatography column, 2.1 mm x 250 mm at 40° C. The LC mobile phase consisted of A) water containing 0.3% formic acid and 10 mM ammonium formate and B) methanol containing 0.3% formic acid and 10 mM ammonium formate.

The gradient elution was linear from 5% B to 45% B, 0-8.5 min, linear from 45% B to 90% B, 8.5-9 min, isocratic from 90% B to 90% B, 9-12.5 min, linear from 90% B to 5% B, 12.5-13 min, with a 4 min re-equilibration period between runs. The flow rate was 0.27 mL/min, and PDA absorbance was monitored from 210 nm to 400 nm. All parameters of the ESI-MS were optimized and selected based on generation of protonated molecular ions ([M + H]+) of the analytes of interest, and production of characteristic fragment ions. The following instrumental parameters were used for LC/MS/MS Multiple Reaction Monitoring (MRM) analysis of monatin and tryptophan: Capillary: 3.5 kV; Cone: 40 V; Hex 1: 20 V; Aperture: 0 V; Hex 2: 0 V; Source temperature: 100° C; Desolvation temperature: 350° C; Desolvation gas: 500 L/h; Cone gas: 50 L/h; Low mass resolution (Q1): 12.0; High mass resolution (Q1): 12.0; Ion energy: 0.2; Entrance: -5 V; Collision Energy: 8; Exit: 1 V; Low mass resolution (Q2): 15; High mass

resolution (Q2): 15; Ion energy (Q2): 3.5; Multiplier: 650. Four monatin-specific parent-to-daughter MRM transitions and one tryptophan specific parent-to-daughter transition are used to specifically detect monatin and tryptophan in *in vitro* and *in vivo* reactions. The transitions monitored are 293.08 to 157.94, 293.08 to 167.94, 293.08 to 130.01, and 293.08 to 256.77. Tryptophan is monitored with the MRM transition 205.0 to 146.0. For internal standard quantification of monatin and tryptophan, four calibration standards containing four different ratios of each analyte to d_5-tryptophan and d_5-monatin, are analyzed. These data are subjected to a linear least squares analysis to form a calibration curve for monatin and tryptophan. To each sample is added a fixed amount of d_5-tryptophan and d_5-monatin (d_5-monatin was synthesized from d_5-tryptophan according to the methods from WO 2003/091396 A2), and the response ratios (monatin/d_5-monatin; tryptophan/d_5-tryptophan) in conjunction with the calibration curves described above are used to calculate the amount of each analyte in the mixtures. Parent-to-daughter mass transitions monitored for d_5-tryptophan and d_5-monatin are 210.0 to 150.0, and 298.1 to 172.0 and 298.1 to 162.00 respectively.

Chiral LC/MS/MS (MRM) Measurement of Monatin

Determination of the stereoisomer distribution of monatin in biochemical reactions was accomplished by derivatization with 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide (FDAA), followed by reversed-phase LC/MS/MS MRM measurement.

Derivatization of Monatin with FDAA

To 50 μL of sample or standard and 10 μL of internal standard was added 100 μL of a 1% solution of FDAA in acetone. Twenty μL of 1.0 M sodium bicarbonate was added, and the mixture was incubated for 1 h at 40° C with occasional mixing. The sample was removed and cooled, and neutralized with 20 μL of 2.0 M HCl (more HCl may be required to effect neutralization of a buffered biological mixture). After degassing was complete, samples were ready for analysis by LC/MS/MS.

LC/MS/MS Multiple Reaction Monitoring for the Determination of the Stereoisomer Distribution of Monatin

Analyses were performed using the LC/MS/MS instrumentation described in the previous sections. The LC separations capable of separating all four stereoisomers of monatin (specifically FDAA-monatin) were performed on a Phenomenex Luna® 2.0 x 250 mm (3 μm) C18 reversed phase chromatography column at 40°C. The LC mobile phase consisted of A) water containing 0.05% (mass/volume) ammonium acetate and B) acetonitrile. The elution was isocratic at 13% B, 0-2 min, linear from 13% B to 30% B, 2-15 min, linear from 30% B to 80% B, 15-16 min, isocratic at 80% B 16-21 min, and linear from 80% B to 13% B, 21-22 min, with a

8 min re-equilibration period between runs. The flow rate was 0.23 mL/min, and PDA absorbance was monitored from 200 nm to 400 nm. All parameters of the ESI-MS were optimized and selected based on generation of deprotonated molecular ions ($[M - H]^-$) of FDAA-monatin, and production of characteristic fragment ions.

The following instrumental parameters were used for LC/MS analysis of monatin in the negative ion ESI/MS mode: Capillary: 3.0 kV; Cone: 40 V; Hex 1: 15 V; Aperture: 0.1 V; Hex 2: 0.1 V; Source temperature: 120°C; Desolvation temperature: 350°C; Desolvation gas: 662 L/h; Cone gas: 42 L/h; Low mass resolution (Q1): 14.0; High mass resolution (Q1): 15.0; Ion energy: 0.5; Entrance: 0 V; Collision Energy: 20; Exit: 0 V; Low mass resolution (Q2): 15; High mass resolution (Q2): 14; Ion energy (Q2): 2.0; Multiplier: 650. Three FDAA-monatin-specific parent-to-daughter transitions were used to specifically detect FDAA-monatin in *in vitro* and *in vivo* reactions. The transitions monitored for monatin were 542.97 to 267.94, 542.97 to 499.07, and 542.97 to 525.04. Monatin internal standard derivative mass transition monitored was 548.2 to 530.2. Identification of FDAA-monatin stereoisomers was based on chromatographic retention time as compared to purified monatin stereoisomers, and mass spectral data. An internal standard was used to monitor the progress of the reaction and for confirmation of retention time of the S,S stereoisomer.

Liquid Chromatography-Post Column Fluorescence Detection of Amino Acids, including Tryptophan, Monatin, Alanine, and HMG

Procedure for Trytophan, Monatin, and Alanine

Liquid chromatography with post-column fluorescence detection for the determination of amino acids in biochemical reactions was performed on a Waters 2690 LC system or equivalent combined with a Waters 474 scanning fluorescence detector, and a Waters post-column reaction module (LC/OPA method). The LC separations were performed on an Interaction-Sodium loaded ion exchange column at 60°C. Mobile phase A was Pickering Na 328 buffer (Pickering Laboratories, Inc.; Mountain View, CA). Mobile phase B was Pickering Na 740 buffer. The gradient elution was from 0% B to 100% B, 0-20 min, isocratic at 100% B, 20-30 min, and linear from 100% B to 0% B, 30-31 min, with a 20 min re-equilibration period between runs. The flow rate for the mobile phase was 0.5 mL/min. The flow rate for the OPA post-column derivatization solution was 0.5 mL/min. The fluorescence detector settings were EX 338 nm and Em 425 nm. Norleucine was employed as an internal standard for the analysis. Identification of amino acids was based on chromatographic retention time data for purified standards.

Procedure for HMG

Samples from biochemical reactions were cleaned up by solid phase extraction (SPE) cartridges containing C18 as the packing material and 0.6% acetic acid as the eluent. The

collected fraction from SPE was then brought up to a known volume and analyzed using HPLC post-column O-Phthaladehyde (OPA) derivatization with a florescensce detector. Chromatographic separation was made possible using a Waters 2695 liquid chromatography system and two Phenomenex AquaC18 columns in series; a 2.1 mm x 250 mm column with 5 μm particles, and a 2.1 mm x 150 mm column with 3 μm particles. The temperature of the column was 40°C and the column isocratic flow rate was 0.18 mL/min. The mobile phase was 0.6% acetic acid. OPA post-column derivatization and detection system consists of a Waters Reagent Manager (RMA), a reaction coil chamber, a temperature control module for the reaction coil chamber, and a Waters 2847 Florescent detector. The OPA flow rate was set at 0.16 mL/min, and the reaction coil chamber was set to 80°C. The florescensce detector was set with an excitation wavelength of 348 nm and an emission wavelength of 450 nm. Other parameters controlling detector sensitivity, such as signal gain and attenuation, were set to experimental needs. Quantification of HMG was based off of the molar response of glutamic acid.

Detection of MP by LC/MS

Liquid chromatography separations were made using Waters 2690 liquid chromatography system and a 2.1 mm x 50 mm Agilent Eclipse XDB- C18 1.8 μm reversed-phase chromatography column with flow rate at 0.25 mL/min and gradient conditions as follows:

Time (min)	A%	B%
0.00	95	5
0.2	95	5
1.2	5	95
4.5	5	95
5.0	95	5
10	95	5

The mobile phase A was 0.3% (v/v) formic acid with 10 mM ammonium formate, and mobile phase B was 0.3% formic acid w/ 10 mM ammonium formate in 50:50 methanol/acetonitrile. The column temperature was 40° C.

Parameters for the Micromass ZQ quadrupole mass spectrometer operating in negative electrospray ionization mode (-ESI) were set as follows: Capillary: 2.2 kV; Cone: 35 V; Extractor: 4 V; RF lens: 1 V; Source temperature: 120°C; Desolvation temperature: 380°C; Desolvation gas: 600 L/h; Cone gas: Off; Low mass resolution: 15.0; High mass resolution: 15.0; Ion energy: 0.2; Multiplier: 650. Single ion monitoring MS experiment was set up to allow detection selectively for m/z 290.3, 210.3, 184.3, and 208.4. The m/z 208.4 is the deprotonated molecular $[M-H]^-$ ion of the internal standard d_5-tryptophan.

Detection of MP by LC/MS/MS

LC separations were made using Waters HPLC liquid chromatography system and a 2.1mm x 50 mm Agilent Eclipse XDB- C18 1.8 μm reversed-phase chromatography column with flow rate at 0.25 mL/min and gradient conditions are as follows:

Time (min)	A%	B%
0.00	95	5
0.7	95	5
3.0	5	95
4.0	5	95
4.3	95	5
6.0	95	5

Mobile phase A was 0.3% (v/v) formic acid with 10 mM ammonium formate, and B was 0.3% formic acid with 10 mM ammonium formate in 50:50 methanol/acetonitrile. The column temperature was 40°C.

Parameters on Waters Premier XE triple quadrupole mass spectrometer for LC/MS/MS Multiple Reaction Monitoring (MRM) experiments operating in negative electrospray ionization mode (-ESI) were set as the following; Capillary: 3.0 kV; Cone: 25 V; Extractor: 3 V; RF lens: 0 V; Source temperature: 120°C; Desolvation temperature: 350°C; Desolvation gas: 650 L/hr; Cone gas: 47 L/hr; Low mass resolution (Q1): 13.5; High mass resolution (Q1): 13.5; Ion energy (Q1): 0.5 V; Entrance: 1 V; Collision Energy: 18 V; Exit 1: 19; Low mass resolution (Q2): 15; High mass resolution (Q2): 15; Ion Energy (Q2): 2.0; Multiplier: 650. Four parent-to-daughter MRM transitions were monitored to selectively detect Monatin precursor (MP) and d_5-Monatin precursor (d_5-MP); d_5-MP was used as an internal standard (I.S.). The four MRM transitions were 290.1 to 184.1, 290.1 to 210.1, 290.1 to 228.1, and 295.1 to 189.1. Two of these transitions, 290.1 to 184.1 for MP, and 295.1 to 189.1 for d_5-MP, were used for generating calibration curves and for quantification purposes. Transitions of 290.1 to 210.1 and 290.1 to 228.1 were used as qualitative secondary confirmation of MP.

Production of Monatin and MP for Standards and for Assays

Production of Monatin

A racemic mixture of R,R and S,S monatin was synthetically produced as described in U.S. Patent No. 5,128,482. The R,R and S,S monatin were separated by a derivatization and a hydrolysis step. Briefly, the monatin racemic mixture was esterified, the free amino group was blocked with carbamazepine (CBZ), a lactone was formed, and the S,S lactone was selectively hydrolyzed using an immobilized protease enzyme. The monatin can also be separated as described in Bassoli et al., *Eur. J. Org. Chem.*, 8:1652-1658, (2005).

MP Production

R-MP was produced by the transamination of R,R monatin using AT-103 broad range D-aminotransferase (BioCatalytics, Pasadena, CA) in 0.1 M potassium phosphate buffer, using sodium pyruvate as the amino acceptor. S-MP was produced by the transamination of S,S monatin using AT-102 L-aminotransferase (BioCatalytics) in 0.1 M potassium phosphate buffer, using sodium pyruvate as the amino acceptor. Both reactions were carried out at 30°C and at a pH of approximately 8.0-8.3, for approximately 20 hours. Both compounds were purified using preparative scale HPLC with a Rohm and Haas (Philadelphia, PA) hydrophobic resin (XAD™1600), eluting in water. Samples containing greater than 90% purity monatin precursor were collected and freeze-dried.

Example 4—Protein Preparation Methods

This example describes the methodology used for cloning, expression, cell extract preparation, protein purification, and protein quantification for secondary characterization of selected DATs.

Those of skill in the art would realize that the presence of activity in a polypeptide encoded from a subcloned (e.g., a fragment) or otherwise modified (e.g., tagged) nucleic acid is considered predictive of the presence of activity in the corresponding polypeptide encoded from the full-length or wild type nucleic acid.

Amplification of DAT-encoding genes for cloning into Topo plasmids

PCR reactions for Topo cloning (using either Pfu Turbo or Cloned Pfu from Stratagene) were as follows: 1X recommended buffer for the polymerase enzyme, 0.2 mM dNTPs, 0.5 μM of each primer, and 1 μl per 50 μl of reaction of the polymerase (2.5 units). The reactions contained approximately 5-100 ng of template DNA per reaction. A 94°C hot start for 2 minutes was used for PCRs, as well as a melting temperature of 94°C. The annealing temperature was dependent on the Tm of the primers, and was either 30 or 60 seconds. The extension time (at 72°C) was at least 2 min per kb. The reaction products were normally separated on a 1x TAE 1% agarose gel, and bands of appropriate sizes were purified with QIAquick Gel Extraction Kit as recommend by the manufacturer except an elution volume of 10 to 50 μl was used. Volumes of 1 to 4 μl of the purified PCR product were used for ligation with the pCRII-Topo Blunt plasmid (Invitrogen, Carlsbad, CA) as recommended by the manufacturer.

Cloning of DATs in pET30a for untagged expression

The DATs having the sequence shown in SEQ ID NO:945, 947, 949 , 891, 893, 869, 873, 877, 881, 883, and 895 (encoding the polypeptides having the sequence of SEQ ID NO:946, 948, 950 , 892, 894, 870, 874, 878, 882, 884, and 896) were amplified from plasmids or PCR products

with Pfu Turbo (Stratagene, La Jolla, CA) and primers adding a *Nde* I at the 5' end and either a *Not* I or *BamH* I restriction site at the 3' end. The PCR fragments were cloned into pCR-Blunt II-Topo (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. The sequence was verified by sequencing (Agencourt, Beverly, MA) and inserts with the correct sequences were then released from the vector using the appropriate restriction enzymes and ligated into the *Nde* I and *Not* I (or *BamH* I) restriction sites of pET30a. See Table 2 for specific primers.

The DAT nucleic acid having the sequence of SEQ ID NO:155 (encoding the polypeptide having SEQ ID NO:156) was amplified with Pfu Turbo (Stratagene) and primers adding a *Nde* I and *Hind* III restriction site at the 5' and 3' end, respectively. The PCR fragments were digested using *Nde* I and *Hind* III restriction enzymes and ligated into the *Nde* I and *Hind* III restriction sites of pET30a. See Table 2 for specific primers. It should be noted that the polypeptide having the sequence of SEQ ID NO:156 appeared to contain the following leader sequence with a probability of 0.991 (as determined by SignalP, as discussed in Nielsen, 1997, *Protein Engineering*, 10:1-6): KNSPIIAAYRAATPGSAAA (SEQ ID NO:1084). The nucleic acid encoding this DAT polypeptide was cloned with the apparent leader sequence.

Table 2. Primers for amplification

Amplifies SEQ ID NO	PCR primers	SEQ ID NO:
945	5'-CCGCCCCATATGAACGCACTAGGATATTACAACGGAAAATGG-3' 5'- GGCGGATCCTTATCCAAAGAATTCGGCACGAGCTGTC-3'	978 979
947	5'- CCGCCCCATATGCGCGAAATTGTTTTTTTGAATGGG -3' 5'- CGGATCCCTAAACCATCTCAAAAAACTTTTGCTGAATAAACCGTG -3'	980 981
949	5'-CCGCCCCATATGTTGGATGAACGGATGGTGTTCATTAAC-3' 5'-GGCGGATCCCTAGTCCACGGCATAGAGCCACTCGG-3'	982 983
891	5'-GGCCGCATATGGACGCACTGGGATATTACAACGGAAAATG-3' 5'- GGCCGCGGCCGCCTATGCCTTTCTCCACTCAGGCGTGTAGC -3'	984 985
893	5'- GGCCGCATATGGACGCACTGGGATATTACAACGGAAAATG -3' 5'- GGCCGCGGCCGCCTATACTGTGCTCCACTCAGGCGTGTAGCC -3'	986 987
869	5'- CATATGTATTCATTATGGAATGATCAAATAGTGAAGG -3' 5'- GCGGCCGCCTATTTATTCGTAAAAGGTGTTGGAATTTTCG -3'	988 989
873	5'- CATATGAGCACCCCGCCGACCAATC -3' 5'- GCGGCCGCCTAGGCCGCCTTCACTTCACGCTC -3'	990 991
877	5'- CATATGAGCACCCCGCCAACCAATTC -3' 5'- GCGGCCGCCTACGCGGCCTTCACTTCGCGC -3'	992 993
881	5'- TCCAGGCATATGAGCACAGTATATTTAAATGGCC -3' 5'- CCAGTAGCGGCCGCCTAACACTCAACACTATACTTATGC -3'	994 995
883	5'- TCTAGGCATATGGTTTATCTGAACGGGCG -3' 5'- ACTGTAGCGGCGGCCTATCCGAGGGACGCGTTGG -3'	996 997
895	5'- CATATGAAAGAGCTGGGCTATTACAACGGAAAAATC-3' 5'- GCGGCCGCCTATGACCTCCACCCCTGATTTCCAAAATAC-3'	998 999
155	5'-CTAGGATTCCATATGAAGAATTCGCCGATCATC-3' 5'-CGAAGCTTCAACAGCGGCCGCTTAAAG -3'	1000 1001

Site-directed mutagenesis

Site-directed mutagenesis was performed using QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. To generate the SEQ ID NO:870 T242N mutant, the pET30a untagged construct described in Example 10 was used as the template. To generate SEQ ID NO:220 G240N and SEQ ID NO:220 T241N mutants, the pET30a construct with a C-terminal his tag described in Example 10 was used as the template. The mutagenic primers used are listed below in Table 3. All the desired mutations were confirmed by DNA sequencing.

Table 3. Primer sequences

Mutant polypeptide designation (SEQ ID NO)	Sequence	SEQ ID NO:
870 T242N	5'- AATTATTTGTTTCATCAACAAATTCTGAAATTACGCCGGTTATTG-3'	1002
220 G240N	5'- CTTGTGTCCAGCAGCAACACACTCGGCCTTAG -3'	1003
220 T241N	5'- GTCCAGCAGCGGCAACCTCGGCCTTAGCGCC -3'	1004

Cloning of DAT PCR products in pET30a for the expression as untagged protein

DAT nucleic acids having the sequences shown in SEQ ID NO:177, 179, 153, 165, 181, 217, 187, 189, 207, 219, 215, 195, 199, 197, 209, 201, 221, 235, 203, 237, 239, 223, 225, 227, 229, 231, 245, 213, 155, 169, 171, 167, 173, and 175 (encoding DAT polypeptides having the sequence shown in SEQ ID NO:178, 180, 154, 166, 182, 218, 188, 190, 208, 220, 216, 196, 200, 198, 210, 202, 222, 236, 204, 238, 240, 224, 226, 228, 230, 232, 246, 214, 156, 170, 172, 168, 174, and 176) were received as PCR products with *Nde* I and *Not* I compatible ends, as well as extraneous nucleotides to improve cutting efficiencies.

The DAT PCR products contained an *Nde*I restriction enzyme site at the 5' end and a *Not*I site at the 3' end. The PCR fragments were first cloned into pCR4 TOPO or pCR-Blunt II-TOPO vector (Invitrogen). After the DNA sequences were verified by sequencing, the DAT genes were released from the TOPO plasmids by the digestion of *Nde*I and *Not*I and ligated into the pET30a vector which had been cut using the same restriction enzymes. DAT genes containing either an *Nde*I or *Not*I site internally were amplified using primers with compatible restriction enzyme sites and cloned into pET30a. For example, the DAT nucleic acid having the SEQ ID NO:155 (encoding the polypeptide having the sequence of SEQ ID NO:156) was reamplified from the original PCR product using *Nde*I and *Hind*III restriction sites for cloning into pET30a.

Cloning of DATs in pET30a for the expression as the C-His-tagged fusion protein

Nucleic acids encoding DAT 4978 and DAT 4978 T243N (described in Example 6), SEQ ID NO:870 (expressed from plasmid pSE420-cHis), and SEQ ID NO:870 T242N, SEQ ID NO:176 and SEQ ID NO:220 (untagged versions expressed from pET30) were re-amplified with *Pfu* Turbo (Stratagene) and primers that placed an *Xho*I site immediately upstream of the stop codons. PCR fragments were cloned into pCR-Blunt II-Topo (Invitrogen, Carlsbad, CA) as recommended by the manufacturer or directly cloned into the *Nde* I and *Xho* I restriction sites of pET30a The sequence was verified by sequencing (Agencourt, Beverly, MA) and an insert with the correct sequence was then released from the vector using *Nde* I and *Xho* I restriction enzymes and the insert was ligated into the *Nde* I and *Xho* I restriction sites of pET30a. See Table 4 for specific primers and plasmids names.

Table 4. Primer sequences

Polypeptide designation (SEQ ID NO)	Sequence	SEQ ID NO
870 and 870 T242N	5'- CATATGTATTCATTATGGAATGATCAAATAGTGAAGG -3' 5'- CTCGAGTTTATTCGTAAAAGGTGTTGGAATTTTCGTTTC -3'	1005 1006
DAT4978 and DAT4978T243N	5'- CATATGAGTTATAGCTTATGGAATGACCAAATTGTGAATG -3' 5'- CTCGAGTGCGCGAATACCTTTTGGGATTTTCGTATC -3'	1007 1008
220	5'- CTAGGATCTCATATGGACGCACTGGGATATTAC -3' 5'- GCCTCGAGTACCCTGCTCCACTCAGG -3'	1009 1010
176	5'- CTAGGATTCCATATGGACGCGCTTGGCTATTAC -3' 5'- GCCTCGAGTACCCTGCTCCACGCAG -3'	1011 1012

Cloning of CbDAT and CaDAT

A *Clostridium beijerinckii* D-amino-transferase was PCR amplified using Pfu Turbo (Stratagene) and *C. beijerinckii* genomic DNA with PCR primers containing a 5' *Nde*I and a 3' *Not*I restriction site. Genomic DNA was extracted from *C. beijerinckii* (ATCC 51743) using the Purrgene genomic DNA purification kit (Gentra Systems, Minneapolis, MN) per the manufacturer's instructions.

The 824 bp PCR product was gel extracted using a Qiagen Gel Extraction Kit and TOPO cloned into pCR-Blunt II-Topo (Invitrogen). After verifying the sequence, the gene was ligated to *Nde* I / *Not* I cut pET28b and pET30a vectors using a Rapid Ligation kit (Roche).

The *C. acetobutylicum* DAT was amplified by PCR using genomic DNA (ATCC 824) and the Stratagene Optiprime PCR Kit with PCR primers containing a 5' *Nde*I and a 3' *Not*I restriction site. The successful PCR reaction was cloned into the pCR4 TOPO vector and TOPO clones were sequenced. A positive TOPO clone was digested with restriction enzymes *Nde*I and *Not*I and the DAT fragment ligated into pET30a vector digested with the same restriction enzymes.

Table 5. Primer sequences

Designation	Sequence	SEQ ID NO:
CbDAT1	5'- GGTTCATATGGAGAATTTAGGTTATTA -3' 5'- GGAAGCGGCCGCATATTCTACCTCCTATTCTG -3'	1013 1014
CaDAT2	5'- GGTTCATATGAAAGATTTAGGATATTACAATGGAGAATAC-3' 5'GGAAGCGGCCGCTTAATTTGTTTCTTCCAAAAATTCATTAAG -3'	1015 1016

In vitro synthesis of LsDAT

The *Lactobacillus salivarius* DAT was assembled using a revised method based on Stemmer et al., 1995, *Gene*, 164:49-53. Briefly, 43 oligonucleotides (primarily 40 mers) were ordered from IDT based on the gene sequence and its complementary DNA sequence, with 20 basepair overlaps between the sense and antisense strands. See Table 6 for the primer list. The primers were diluted to 250 μM in water and 5 μL of each primer was mixed together in a microfuge tube. PCR was carried out as follows: per 100 μL reaction, 1.5 μL of the primer pool, 4 μL dNTPs, 1X XL PCR buffer, 1 mM magnesium acetate, 2 μL r*Tth* polymerase (Roche, Indianapolis, IN), and 0.25 μL Pfu polymerase (Stratagene, La Jolla, CA) were added. A 3 minute hot start was done at 94°C, followed by 15 cycles of 94°C for 30 seconds, 42°C for 30 seconds, and 68°C for 15 seconds. Ten more cycles were done with an extension time of 30 seconds (at 68°C). Ten more cycles were performed with an extension time of 75 seconds. Lastly, a chain extension step was done for seven minutes at 68°C.

Table 6. Oligos used to synthesis LsDAT

Designation	Sequence (5' → 3')	SEQ ID NO:
F1:	ATGAAGCAAG TTGGATACTA CAATGGTACT ATCGCTGATT	1017
F2:	TAAATGAACT TAAGGTGCCT GCTACTGATC GTGCACTTTA	1018
F3:	TTTTGGTGAT GGTTGCTACG ATGCAACTAC ATTTAAGAAC	1019
F4:	AATGTTGCAT TTGCCTTAGA AGATCATCTT GATCGTTTTT	1020
F5:	ATAATAGTTG TCGCCTACTA GAGATCGATT TCCCTTTAAA	1021
F6:	TCGCGATGAA CTTAAAGAAA AGCTTTACGC TGTTATTGAT	1022
F7:	GCTAACGAAG TTGATACTGG TATCCTTTAT TGGCAAACTT	1023
F8:	CACGTGGTTC TGGTTTACGT AACCATATTT TCCCAGAAGA	1024
F9:	TAGCCAACCT AATTTATTAA TTTTTACTGC TCCTTATGGT	1025
F10:	TTAGTTCCAT TTGATACTGA ATATAAACTT ATATCTCGCG	1026
F11:	AAGACACTCG CTTCTTACAT TGCAATATTA AAACTTTGAA	1027
F12:	TTTACTTCCA AACGTTATTG CAAGTCAAAA GGCTAATGAA	1028
F13:	AGTCATTGCC AAGAAGTGGT CTTCCATCGC GGTGACAGAG	1029
F14:	TTACAGAATG TGCACACTCT AACATCTTAA TTCTAAAAGA	1030
F15:	TGGCGTTCTT TGCTCCCCAC CTAGAGATAA TTTAATCTTG	1031
F16:	CCAGGAATTA CTTTGAAACA TCTCTTGCAA TTAGCAAAAG	1032
F17:	AAAATAATAT TCCTACTTCC GAAGCACCAT TCACTATGGA	1033
F18:	TGATCTTAGA AATGCTGATG AAGTTATTGT TAGTTCTTCA	1034
F19:	GCTTGTCTAG GTATTCGCGC AGTCGAGCTT GATGGTCAGC	1035
F20:	CTGTTGGTGG AAAAGATGGA AAGACTTTAA AGATCTTGCA	1036
F21:	AGATGCTTAT GCTAAGAAAT ATAATGCTGA AACTGTAAGT CGTTAA	1037
R1:	TAGTATCCAA CTTGCTTCAT	1038
R2:	AGGCACCTTA AGTTCATTTA AATCAGCGAT AGTACCATTG	1039

R3:	CGTAGCAACC ATCACCAAAA TAAAGTGCAC GATCAGTAGC	1040
R4:	TCTAAGGCAA ATGCAACATT GTTCTTAAAT GTAGTTGCAT	1041
R5:	TAGTAGGCGA CAACTATTAT AAAAACGATC AAGATGATCT	1042
R6:	TTTCTTTAAG TTCATCGCGA TTTAAAGGGA AATCGATCTC	1043
R7:	CCAGTATCAA CTTCGTTAGC ATCAATAACA GCGTAAAGCT	1044
R8:	ACGTAAACCA GAACCACGTG AAGTTTGCCA ATAAAGGATA	1045
R9:	TTAATAAATT AGGTTGGCTA TCTTCTGGGA AAATATGGTT	1046
R10:	TCAGTATCAA ATGGAACTAA ACCATAAGGA GCAGTAAAAA	1047
R11:	ATGTAAGAAG CGAGTGTCTT CGCGAGATAT AAGTTTATAT	1048
R12:	CAATAACGTT TGGAAGTAAA TTCAAAGTTT TAATATTGCA	1049
R13:	ACCACTTCTT GGCAATGACT TTCATTAGCC TTTTGACTTG	1050
R14:	AGAGTGTGCA CATTCTGTAA CTCTGTCACC GCGATGGAAG	1051
R15:	GTGGGGAGCA AAGAACGCCA TCTTTTAGAA TTAAGATGTT	1052
R16:	TGTTTCAAAG TAATTCCTGG CAAGATTAAA TTATCTCTAG	1053
R17:	GGAAGTAGGA ATATTATTTT CTTTTGCTAA TTGCAAGAGA	1054
R18:	CATCAGCATT TCTAAGATCA TCCATAGTGA ATGGTGCTTC	1055
R19:	GCGCGAATAC CTAGACAAGC TGAAGAACTA ACAATAACTT	1056
R20:	TCCATCTTTT CCACCAACAG GCTGACCATC AAGCTCGACT	1057
R21:	ATTTCTTAGC ATAAGCATCT TGCAAGATCT TTAAAGTCTT	1058
R22:	TTAACGACTT ACAGTTTCAG CATTAT	1059

A secondary amplification with primers *L. sal* DAT R *Not* I and *L. sal* DAT F *Nde* I (below) resulted in a band of the correct molecular weight. See Table 7 for these secondary amplification primer sequences.

Table 7. Primer sequences

Designation	Sequence (5' → 3')	SEQ ID NO:
L. sal DAT R NotI	TTGGCCAAGCGGCCGCTTAACGACTTACAGTTT	1060
L. sal DAT F NdeI	GGTTCCAAGGCATATGAAGCAAGTTGGATACTA	1061

The secondary PCR reaction was set up the same as above with the exception that only 2 primers were added. For the PCR template, 2.5 µl of the primary PCR reaction was used. A 3 minute hot start was done at 94°C, followed by 10 cycles of 94°C for 30 seconds, 42°C for 30 seconds, and 68°C for 15 seconds. Ten more cycles were done with an increased annealing temp of 48°C for 30 seconds with an extension time of 30 seconds (at 68°C). Lastly, a chain extension step was done for seven minutes at 68°C.

The fragment was cloned into a pCR-BluntII-TOPO vector and the TOPO clones were sequenced. A positive TOPO clone was cut with *Nde*I and *Not*I and the DAT fragment ligated into pET30a vector digested with the same restriction enzymes.

Enzyme preparation

E. coli strain BL21(DE3) was used as the host strain for the expression of DATs from pET-derived plasmids. *E. coli* strain TOP10 was used in all other DAT consructs. Single colonies of desired constructs were typically innoculated into Overnight Express II medium (Novagen) containing the appropriate amount of antibiotics. Following cultivation at 30°C

overnight, the cells were harvested by centrifugation when the OD_{600} was greater than 10. Alternatively, overnight cultures were utilized to innoculate cultures in LB medium containing the appropriate antibiotics. The cultures were grown at 30°C to an OD_{600} of 0.5 to 0.9 and protein expression was induced with 1 mM IPTG for 4 h at the same temperature.

5 Cell extracts were prepared by adding 5 mL per g of cell pellet or 5 mL per 50 mL of overnight culture, of BugBuster® (primary amine-free) Extraction Reagent (EMD Biosciences/Novagen catalog #70923) with 5 μL/mL of Protease Inhibitor Cocktail II (EMD Bioscience/Calbiochem catalog #539132), 1 μl/ml of Benzonase® Nuclease (EMD Biosciences/Novagen catalog #70746), and 0.033 μl/ml of r-LysozymeTM solution (EMD
10 Biosciences/Novagen catalog #71110) to the cells. The cell resuspension was incubated at room temperature for 15 min with gentle shaking. Following centrifugation at 16,100 rcf for 20 min at 4°C, the supernatant was removed as the cell-free extract.

Prior to using the enzyme preparation for monatin reactions, detergents and low molecular weight compounds were removed from the cell-free extract by passage through a PD-
15 10 column (GE Healthcare, Piscataway, NJ) that was previously equilibrated with potassium phosphate buffer (100 mM, pH 7.8) or EPPS buffer (100 mM, pH 8.2) containing 0.05 mM of PLP. The protein was eluted using the equilibration buffer. Protein concentrations were typically determined using the BioRad Coomassie plate assay (also known as the Bradford assay) plate assay with BSA (Pierce) as the standard. Occasionally, the BCA (Pierce) microtiter
20 plate assay was used for protein determination, where noted. To estimate the concentration of the D-aminotransferase in the cell-free extracts, 1 mg/mL samples were loaded on the Experion (Bio-Rad, Hercules, Ca) electrophoresis system and the Experion Software (Version 2.0.132.0) was used to calculate the percentage of the soluble DAT protein in the cell-free extract. Alternatively, SDS-PAGE analysis was done and visual estimation was used to estimate
25 percentage of expression.

The His-tagged fusion proteins were purified using either the GE Healthcare Chelating Sepharose Fast Flow resin or Novagen His-Bind columns. The purification using the Sepharose resin involved loading the cell-free extract onto a column that was previously equilibrated with potassium phosphate buffer (100 mM, pH 7.8) containing 200 mM of sodium chloride and 0.050
30 mM of PLP. The column was then washed successively using 3-5 column volumes of the equilibration buffer, 3-5 column volumes of the equilibration buffer containing 25 mM of imidazole and 3-5 column volumes of the equilibration buffer containing 50-100 mM of imidazole. The His-tagged protein was eluted off the column using 3-5 column volumes of the equilibration buffer containing 500 mM of imidazole. The eluate was concentrated using the
35 Amicon (Billerica, MA) Centricon-70. The imidazole and sodium chloride salts in the

concentrated protein solution were removed by passage through PD-10 desalting columns that were previously equilibrated using potassium phosphate buffer (100 mM, pH 7.8) (for DAT4978 and DAT4978 T243N) or EPPS buffer (100 mM, pH 8.2) (for SEQ ID NO:870 and SEQ ID NO:870 T242N) containing 50 μM of PLP. Protein concentrations were determined using Bio-Rad Protein Assay (Bio-Rad) and Albumin (Pierce) as a standard. Aliquots (0.5-1 mL) of the purified enzyme were stored at –80°C until use. The purification of the His-tagged protein using the His-Bind columns followed the manufacture's instruction. The eluate from the column was desalted using the PD10 column as described above.

Example 5—Assay Procedures #2 for D-aminotransferase activity

Monatin Production Assay (Standard)

The following components were combined: 100 mM EPPS, pH 8.2; 200 mM sodium pyruvate; 100 mM of D-tryptophan; 50 μM PLP; 1 mM $MgCl_2$; 0.01% Tween-80; 50 μg/mL of aldolase described in Example 6 (cell-free extract was used; the aldolase concentration was estimated based on the percentage reading from Experion chip) and an appropriate amount of DAT (typically 0.1-1 mg/mL).

Except for the PLP stock solution and the protein solutions, all other reagents were made using oxygen-free deionized water and stored in the anaerobic chamber. The reactions were set up in the anaerobic chamber at room temperature with constant gentle mixing. To take a time point, formic acid was added into an aliquot of the reaction mixture to a final concentration of 2% (v/v). Following centrifugation at 16,100 RCF for 5 min using a bench-top microfuge, the supernantant was filtered through a 0.2 μm nylon membrane filter. Samples were then diluted 20- to 100-fold with water prior to analysis by LC/MS/MS.

D-tryptophan Transamination Assay

To compare the D-tryptophan transamination activities of certain D-aminotransferases, the following assays were performed. The assay mix contained: 0.5 mg/mL of cellular extract protein containing D-AT; 40 mM potassium phosphate pH 8.0; 20 mM D-tryptophan; 100 mM sodium pyruvate; and 50 μM PLP. The assays were incubated at 37°C for 30 minutes and then placed on ice.

The extent of reaction was followed by measuring the amount of indole-3-pyruvate formed using the following assay: to 5 μl, 10 μl and 20 μl of reaction mix, 200 μl of the following solution was added: 0.5 mM sodium arsenate; 0.5 mM EDTA; and 50 mM sodium tetraborate (pH 8.5). Absorbance of the indole-3-pyruvate enol-borate complex at 325 nm was compared to a standard curve of indole-3-pyruvate prepared in the same solution.

Alanine formation can also be used to follow the extent of the D-tryptophan transamination reactions. Alanine concentrations were determined as described in Example 3.

R, R Monatin Transamination Assay

Assay conditions (final volume 2 mL) included: 0.01% Tween; 100 mM EPPS pH 8.2; 100 mM sodium pyruvate; approximately 3 mM R,R monatin; 0.5 mg/mL DAT; and 50 μM PLP. The extent of reaction was monitored by detection of alanine or R-MP formed using the protocols described in Example 3.

Example 6—Method for obtaining DATs and an aldolase

This method described the cloning of the aldolase used in monatin formation reactions with the D-aminotransferases, and D-aminotransferases previously isolated that were used for comparative purposes.

Aldolase

The aldolase used in monatin production assays from D-tryptophan was isolated and subcloned into pET vectors as described in WO 2007/103389 (referred to in that application as the aldolase of SEQ ID NO:276 encoded by the nucleic acid of SEQ ID NO:275).

DAT and DAT4978 T243N

A D-aminotransferase from ATCC #4978 (DAT 4978) was cloned as described in U.S. Publication No. 2006/0252135. A T243N mutant was made using the pET30 (untagged) DAT 4978 construct.

The primer for mutagenesis was designed following the suggestions listed in the Stratagene Multi-Change kit (La Jolla, CA). The primer was 5'-phosphorylated. Mutagenesis was done using the Stratagene Multi-Change kit following the manufacturer's instructions. The mutagenic oliognucleotide sequence is shown in Table 8.

Table 8. Mutagenic oligonucleotide sequences

Mutant name	Amino acid change	Primer	SEQ ID NO:
DAT4978T243N	T243N	5'-GTGATTGTTTCATCAACGAATTCAGAAGTAACGCC-3'	1062

E. coli XL10-Gold cells (Stratagene) were transformed and the resultant purified plasmid preparations were sequenced to verify that the correct mutations were incorporated. The plasmid containing the DAT 4978 T243N was then transformed into *E. coli* BL21 (DE3) expression host

B. sphaericus DAT

A D-aminotransferase from *B. sphaericus* (ATCC number 10208) was cloned as described in US 2006/0252135. The protein was prepared as described in the same reference.

Example 7—Analysis of DATs

DAT polypeptides having the sequence shown in SEQ ID NO:928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, and 950 were produced by expressing the corresponding nucleic acid in the vectors and in the compatible *E. coli* expression hosts described in Example 2. One skilled in the art can synthesize the genes encoding these D-aminotransferases using assembly PCR techniques such as those described in Example 4. Overnight cultures in LB medium containing carbenicillin (100 μg/mL) were diluted 100X in 100 mL of the same medium and grown in a 500 mL baffled flask. The culture was grown at 30°C to an OD_{600} of 0.5 to 0.9, and protein expression was induced with 1 mM IPTG for 4 h at the same temperature. Samples for total protein were taken immediately prior to harvesting. Cells were harvested by centrifugation and washed once with 10 mL of potassium phosphate buffer pH 7.8. Cells were immediately frozen at –80°C until cell extracts were prepared.

Cell extracts were prepared and desalted as described in Example 4 using 100 mM potassium phosphate as the buffer to elute and equilibrate the PD10 column. Total protein and DAT concentrations were determined as described.

Transamination of R,R monatin with pyruvate as the amino acceptor were performed as described in Example 5 except that 10 mM R,R monatin was utilized. Initial analyses of alanine, monatin, and monatin precursor levels were not consistent with each other and results were considered qualitative. The DAT polypeptide having the sequence of SEQ ID NO:948 appeared to show monatin precursor formation.

For further confirmation of activity, a monatin formation assay was done as described in the methods with a DAT concentration of approximately 0.2 mg/mL. As a control, 0.2 mg/mL concentration of purified *B. sphaericus* DAT was evaluated. After 2 and 21 hr, an aliquot was taken and formic acid was added to a final concentration of 2%, and the samples were frozen. Samples were then thawed, spun and filtered. Samples were analyzed for monatin using LC/MS/MS methodology and for tryptophan and alanine using the LC/OPA post-column fluorescence methodology described in Example 3. The DAT polypeptides having the sequence of SEQ ID NO:946 and 950 were capable of R,R monatin formation under the conditions tested. The DAT polypeptide having the sequence of SEQ ID NO:948 showed a loss of tryptophan and an increase in alanine formation, demonstrating its activity as a D-tryptophan transaminase. The DAT polypeptide having the sequence of SEQ ID NO:946 expressed well as determined by the amount of total protein but was not very soluble, which explains some inconsistent results. The DAT polypeptides having the sequence shown in SEQ ID NO:930, 932, 940, 942, and 944 did not yield visible bands on analysis with SDS-PAGE and, therefore, may be active if produced under different conditions. See Table 9 for results.

Table 9. Activity of DATs

DAT Polypeptide (SEQ ID NO)	Monatin [mM] Time = 2hr	Monatin [mM] Time = 21 hr
928	nd	nd
930	nd	nd
932	nd	nd
934	nd	nd
936	nd	nd
938	nd	nd
940	nd	nd
942	nd	nd
944	nd	nd
946	0.1	0.6
948	nd	nd
950	0.4	3.1
B. sphaericus control DAT	0.8	4.4

nd=not detected under conditions tested

5 Analysis of DAT polypeptides in pET30a

The DAT polypeptides having the sequence of SEQ ID NO:946, 948, and 950 were subcloned into pET30a as described in Example 4. Duplicate cultures of *E. coli* strain BL21 DE3 containing the DATs in pET30a were grown overnight in Overnight Express II (Solution 1-6, Novagen) at both 25 and 30°C. As a control, a strain containing pET30a plasmid without an
10 insert was also grown. Cells were collected at an OD_{600} of 5-10. Cells were harvested by centrifugation and washed once with 10 mL of 100 mM potassium phosphate buffer pH 7.8. Cells were frozen at -80°C until further processed.

Cell extracts were prepared as described in Example 4 using 100 mM potassium phosphate as the buffer to elute and equilibrate the PD10 columns. Total protein and DAT
15 protein concentrations were determined as described. The DAT polypeptide having the sequence of SEQ ID NO:946 expressed well at 30°C in the total protein fraction, but was not soluble as viewed by SDS-PAGE. The DAT polypeptides having the sequence of SEQ ID NO:948 and 950 expressed at the higher temperature also, but were soluble.

A monatin formation assay was done as described in Example 5 except with a DAT
20 concentration of 0.1 mg/mL for the polypeptide of SEQ ID NO:946 (0.5 mg/mL for all others). As a positive control, purified *B. sphaericus* DAT at a 0.5 mg/mL concentration was also assayed. After 2 and 21 hr, an aliquot was taken, formic acid was added to a final concentration of 2%, and the samples were frozen until further processed. Samples were then thawed, spun and filtered. Samples were analyzed for monatin using LC/MS/MS, and for tryptophan and

alanine using LC/OPA post-column fluorescence detection methods described in Example 3. The results are shown in Table 10. D-tryptophan consumption and alanine formation were shown for all the D-aminotransferases tested indicating that they all have activity on D-tryptophan. Under these conditions, only DAT polypeptides having the sequence of SEQ ID NO:946 and 948 appeared to have activity for monatin formation. It is possible that expression or stability differences between the two host systems are the reason why activity is seen in some cases but not in others.

Table 10

DAT polypeptide (SEQ ID NO)	Monatin [mM] time = 2 hr	Monatin [mM] time = 21 hr
pET30 (negative control)	nd	nd
946	0.4	1.8
948	nd	0.2
950	nd	nd
B. sphaericus positive control	1.8	8.6

nd, not detected under conditions tested

Example 8—Analysis of DATs in pSE420-cHis

DAT polypeptides having the sequence shown in SEQ ID NOs:886, 888, 890, 892 and 894 DATs were produced from the pSE420-cHis vector in *E. coli* HMS174. One skilled in the art can synthesize the genes encoding these D-aminotransferases using assembly PCR techniques such as those described in Example 4. Overnight cultures of the various DAT constructs were grown in LB medium containing ampicillin (100 μg/mL) at 30°C. Fifty mL of the same medium was inoculated the next day with 1 mL of the overnight cultures. The cultures were grown at 30°C until the $OD_{600\ nm}$ reached approximately 0.5 and then induced with 1 mM IPTG. The cultures were further incubated for 4 h at 30°C and then harvested by centrifugation at 3800 rcf for 15 min. The cells were washed with 1.5 mL of 50 mM potassium phosphate, pH 7.0 and centrifuged again. The supernatant was decanted and the cell pellets were weighed.

Cell extracts were prepared as described in the methods using 100 mM potassium phosphate as the buffer to elute and equilibrate the column. Total and DAT concentrations were determined as described except BCA (Pierce) was used instead of Bradford for total protein determination. Two different vector only cultures were grown in the same *E. coli* hosts as the cloned DATs. All of the proteins produced visible bands on SDS-PAGE gels, but to differing degrees of solubility. Polypeptides having the sequence of SEQ ID NO:892 were not very soluble.

To compare the D-tryptophan transamination activities of each of the enzymes, the D-tryptophan transamination assay and the R,R monatin transamination assay described in Example 5 were performed. The D-tryptophan aminotransferase targeted using a final concentration of 0.5 mg/mL of cellular extract containing D-aminotransferase and 0.1 mg/mL of the purified *B. sphaericus* DAT as a control. Quantification of the DATs in the cellular extracts was difficult due to the low levels of soluble polypeptides. The DAT polypeptides having the sequence shown in SEQ ID NO:888, 892 and 894 showed good activity with D-tryptophan as a substrate during the 30 minute reaction. DAT polypeptides having the sequence shown in SEQ ID NO:886 and 890 had measurable activity above the no-enzyme control, but exhibited little activity under the conditions tested.

Monatin transamination experiments were performed at room temperature, taking samples after 0.5, 1 and 2 hours targeting 0.5 mg/mL of each DAT, including the purified positive control from *B. sphaericus*. The R,R monatin transamination samples were then analyzed for monatin and alanine. The amount of monatin remaining was quantified by LC/MS/MS; alanine formation was measured using the post-column derivatization method in Example 3. Under the conditions tested, the DAT polypeptides having the sequence shown in SEQ ID NOs:892 and 894 were active. The DAT polypeptide having the sequence of SEQ ID NO:894 appeared to have the highest activity for conversion of R,R monatin to R-MP. The trends were consistent when alanine formation was assayed. The alanine production numbers (in mM) for the various timepoints are shown in Table 11.

Table 11. Alanine formation (mM) from R,R monatin transamination reactions

DAT polypeptide (SEQ ID NO)	0.5 hr	1 hr	2 hr
vector control 1	0.139	0.185	0.215
vector control 2	0.179	0.242	0.301
886	0.128	0.203	0.242
888	0.13	0.203	0.275
890	0.112	0.153	0.176
892	1.034	1.587	2.167
894	2.2	2.52	2.663
BsphDAT(purified)	0.287	0.519	0.894
no enzyme	0.043	0.035	0.037

DAT nucleic acids having the sequence shown in SEQ ID NO:891 and 893 were subcloned into pET30 as described in Example 4. These constructs were transformed into a variety of *E. coli* hosts carrying the DE3 lysogen for expression from a T7 promoter, including both K-12 and B strains of *E. coli*, and one strain that carried the pLysS plasmid. The clones were expressed in OvernightExpress System II as described in Example 4, with and without the

addition of 0.5 mM pyridoxine, and analyzed by SDS-PAGE or Experion for expression. From these experiments, it became apparent that the proteins were expressing mostly in the insoluble fraction. Pyridoxine helped improve solubility to a small degree as did lowering the temperature from 37 to 30°C for induction. Further work was done in cloning systems designed to maximize soluble expression (see Example 16-22).

Example 9—Analysis of *Ca*DAT, *Cb*DAT, and *Ls*DAT in pET30a

The amino acid sequence shown in SEQ ID NO:894 was used to search for similar proteins available in the public databases. Three DATs were found that had similarity to SEQ ID NO:894. They were from *Lactobacillus salivarus* (47% identical at the protein level), *Clostridium beijerinckii* (57% identical at the protein level), and *Clostridium acetobutylicum* (60% identical at the protein level). The gene and protein sequences and their accession numbers are shown at the end of this example. Figure 1 is an alignment showing the consensus regions of these SEQ ID NO:894-like proteins. One can see a high degree of consensus regions indicating structural similarities.

These nucleic acids were cloned into pET30a, and the corresponding polypeptides expressed and tested for activity as described herein.

*Cb*DAT

The D-aminotransferase from *Clostridium beijerinckii* (*Cb*DAT) was cloned into pET30a (untagged) BL21 (DE3) and expressed using Overnight Express II (Novagen). The cells were collected at an optical density at 600 nm of approximately 9 and centrifuged at 4000 rcf for 15 min. The cells were washed once with 100 mM potassium phosphate pH 7.8 (cold), and spun again.

Cell extracts were prepared as described herein using 100 mM EPPS pH 8.2 as the buffer to elute and equilibrate the column. Total protein and DAT protein concentrations were determined as described except the BCA method (Pierce) was used instead of the Bradford (Coomassie) assay. The *Cb*DAT expressed well but was only partially soluble.

A monatin formation assay was done as described in Example 5 but the activity of *Cb*DAT (0.5 mg/mL) was also studied at pH 7.4 (with potassium phosphate as a buffer). As a control, purified *B. sphaericus* DAT (1 mg/mL) was assayed at pH 8.2. After 1, 2, 4, 8, and 23 hrs, aliquots were taken and formic acid was added to a final concentration of 2%. Samples were frozen at –80°C until analyzed. Samples were then thawed, spun and filtered. Samples were analyzed for monatin using the LC/MS/MS methodology described in Example 3. Results are shown in Table 12. The amount of monatin produced were slightly higher for the assays carried out at pH 8.2. Similar experiments were performed with the polypeptides expressed from pET28

with an N-terminal His-tag. The activity of the tagged version appeared to be slightly less than that of the untagged, but still easily detectable.

Table 12. Activity over time

DAT Enzyme	Monatin (ppm) 1 hr	Monatin (ppm) 2 hr	Monatin (ppm) 4 hr	Monatin (ppm) 8 hr	Monatin (ppm) 23 hr
*Cb*DAT pET30 (7.4 mg/mL)	45	126	280	428	502
*Cb*DAT pET30 (8.2 mg/mL)	67	189	344	436	568
B. sphaericus DAT (1 mg/mL)	531	968	1742	2310	3706

5

<u>*Ca*DAT and *Ls*DAT</u>

The D-aminotransferases from *Lactobacillus salivarus* (*Ls*DAT) and *Clostridium acetobutylicum* (*Ca*DAT) were cloned into pET30a (untagged) BL21(DE3) and expressed using Overnight Express II (Novagen). The cells were collected when the culture reached an optical

10 density at 600 nm of approximately 9 by centrifugation at 4000 rcf for 15 minutes.

Cell extracts were prepared as described herein using 100 mM EPPS pH 8.2 as the buffer to elute and equilibrate the column. Total protein and DAT protein concentrations were determined using the BCA (Pierce) protocol. Both enzymes expressed well and were soluble.

The assay was performed at room temperature under anaerobic conditions. As a control,

15 purified *B. sphaericus* D-aminotransferase was assayed. Approximately 0.5 mg/mL of each DAT was used. After 0.5, 1, 2, 4, 6, 8 and 22 hr an aliquot was taken and formic acid added to a final concentration of 2% and the samples were frozen. Samples were then thawed, spun and filtered. Samples were analyzed for monatin using the LC/MS/MS methodology described in Example 3. Results are shown in Table 13.

20

Table 13.

DAT polypeptide	Monatin (ppm) 0.5 hr	Monatin (ppm) 1 hr	Monatin (ppm) 2 hr	Monatin (ppm) 4 hr	Monatin (ppm) 6 hr	Monatin (ppm) 8 hr	Monatin (ppm) 22 hr
B. sphaericus	76.6	194.4	457.6	860.8	1186	1770	2546
*Ls*DAT	2.8	6.4	14.6	33	52	69.8	173
*Ca*DAT	50.2	141.2	318.6	543.4	612	1144	668

The homologs of the DAT having the sequence shown in SEQ ID NO:894 were active. Since the homologs showing the conserved sequence above were all active in monatin formation

25 assays, it is expected that any D-aminotransferase containing the consensus sequences described

herein would also be active, although their primary sequence identity is as low as 47%. There has been no evidence before this work that these unique D-aminotransferases, with low homology to the more characterized *Bacillus* D-aminotransferase, would have activity for monatin or would be broad specificity enzymes.

DNA Sequence *Ca*DAT (ACCESSION AE001437 AE007513-AE007868; VERSION AE001437.1 GI:25168256; nucleotides 914049.. 914891)

```
  1  atgaaagatt taggatatta caatggagaa tacgacttaa ttgaaaatat gaaaatacca
 61  atgaatgatc gtgtatgcta ttttggtgat ggtgtttatg atgctactta tagtagaaac
121  cataatatat ttgcactaga tgagcatatt gaccgatttt ataatagtgc cgagctttta
181  agaattaaaa ttccatatac aaagaaggaa atgaaagagc ttttaaagga tatggttaaa
241  aaggttgata gcggagaaca atttgtatat tggcaggtta ctagaggtac tggcatgcgt
301  aatcatgctt ttttgagtga ggatgttaag gctaatattt ggattgtttt aaagccacta
361  aaggtaaaag atatgtcaaa aaaattaaaa ctaataacat tagaggatac tagattttta
421  cattgtaaca taaaaacctt aaatttgctt cctagtgtaa ttgcagcaca aaaaactgaa
481  gaagcaggct gccaggaagc agtatttcat agaggagata gagttactga atgtgctcat
541  agtaatgttt caattataaa ggatgagatt ttaaaaactg cgccaacaga taatcttatt
601  ttgccgggaa tagcaagggc gcatcttata aaaatgtgca aaaaatttga gatacctgta
661  gatgaaactc catttacatt aaaggagtta attaatgcgg atgaagttat agttacaagt
721  tcagggcaat tttgtatgac tgcttgtgag atagatggaa gacctgtagg cggaaaagcg
781  ccagatatta ttaaaaagct tcagactgcc ttacttaatg aatttttgga agaaacaaat
841  taa  (SEQ ID NO:1063)
```

Protein Sequence *Ca*DAT (ACCESSION NP_347428; VERSION NP_347428.1 GI:15894079)

```
  1  MKDLGYYNGE YDLIENMKIP MNDRVCYFGD GVYDATYSRN HNIFALDEHI DRFYNSAELL
 61  RIKIPYTKKE MKELLKDMVK KVDSGEQFVY WQVTRGTGMR NHAFLSEDVK ANIWIVLKPL
121  KVKDMSKKLK LITLEDTRFL HCNIKTLNLL PSVIAAQKTE EAGCQEAVFH RGDRVTECAH
181  SNVSIIKDEI LKTAPTDNLI LPGIARAHLI KMCKKFEIPV DETPFTLKEL INADEVIVTS
241  SGQFCMTACE IDGRPVGGKA PDIIKKLQTA LLNEFLEETN  (SEQ ID NO:1064)
```

DNA Sequence *Cb*DAT (ACCESSION CP000721 AALO01000000 AALO01000001-AALO01000089 VERSION CP000721.1 GI:149901357; nucleotides 3213484.. 3212636)

```
  1  atggagaatt taggttatta taatggaaag tttggattat tagaggaaat gacagtacca
 61  atgcttgatc gtgtttgcta ttttggagat ggagtttatg atgctactta tagcagaaat
121  cacaagattt ttgcattgga ggagcatatt gaaagatttt acaacagcgc tggtttatta
181  ggaattaaaa ttccttattc aaaggagcaa gtaaaagaaa tccttaagga gatggtatta
241  aaggttgatt caggagaaca atttgtatat tggcaaatta ctagaggaac tggaatgaga
301  aatcatgctt ttcctggaga tgaggtccct gcaaatctat ggattatgtt aaagccttta
361  aatattaagg atatgtcaca aaaattaaag ttaatcactt tagaagacac tagattttta
421  cactgtaata tcaaaacctt aaatttatta ccaagtgtaa ttgcatctca aaaaactgaa
481  gaggcaggat gtcaggaagc tgtatttcat agaggggata gagtaactga atgtgcacat
541  agcaatgtat caattattaa ggatggtata ttaaaaactg ctccaacaga caatttaatt
601  ttaccaggta tagcaagagc tcaccttatt aaaatgtgta aatcctttaa tattcctgta
661  gatgaaacag catttacctt gaaggaatta atggaggcag atgaagttat agttactagt
721  tcaggtcaat tttgtatggc aaccagtgaa atagatggaa tacctgtagg gggaaaagca
781  ccagagcttg taaagaaatt acaagatgca ttgttaaatg agttcttaga agaaacaaaa
841  acagaatag  (SEQ ID NO:1065)
```

Protein Sequence *Cb*DAT (ACCESSION YP_001309869 VERSION YP_001309869.1 GI:150017615)

```
  1  MENLGYYNGK FGLLEEMTVP MLDRVCYFGD GVYDATYSRN HKIFALEEHI ERFYNSAGLL
 61  GIKIPYSKEQ VKEILKEMVL KVDSGEQFVY WQITRGTGMR NHAFPGDEVP ANLWIMLKPL
121  NIKDMSQKLK LITLEDTRFL HCNIKTLNLL PSVIASQKTE EAGCQEAVFH RGDRVTECAH
```

```
181  SNVSIIKDGI LKTAPTDNLI LPGIARAHLI KMCKSFNIPV DETAFTLKEL MEADEVIVTS
241  SGQFCMATSE IDGIPVGGKA PELVKKLQDA LLNEFLEETK TE (SEQ ID NO:1066)
```

DNA Sequence *Ls*DAT (ACCESSION CP000233 VERSION CP000233.1 GI:90820184; nucleotides 1750082.. 1750927)

```
  1  atgaagcaag ttggatacta caatggtact atcgctgatt taaatgaact taaggtgcct
 61  gctactgatc gtgcacttta ttttggtgat ggttgctacg atgcaactac atttaagaac
121  aatgttgcat ttgccttaga agatcatctt gatcgttttt ataatagttg tcgcctacta
181  gagatcgatt tccctttaaa tcgcgatgaa cttaaagaaa agctttacgc tgttattgat
241  gctaacgaag ttgatactgg tatcctttat tggcaaactt cacgtggttc tggtttacgt
301  aaccatattt tcccagaaga tagccaacct aatttattaa tttttactgc tccttatggt
361  ttagttccat ttgatactga atataaactt atatctcgcg aagacactcg cttcttacat
421  tgcaatatta aaactttgaa tttacttcca aacgttattg caagtcaaaa ggctaatgaa
481  agtcattgcc aagaagtggt cttccatcgc ggtgacagag ttacagaatg tgcacactct
541  aacatcttaa ttctaaaaga tggcgttctt tgctccccac ctagagataa tttaatcttg
601  ccaggaatta ctttgaaaca tctcttgcaa ttagcaaaag aaaataatat tcctacttcc
661  gaagcaccat tcactatgga tgatcttaga aatgctgatg aagttattgt tagttcttca
721  gcttgtctag gtattcgcgc agtcgagctt gatggtcagc ctgttggtgg aaaagatgga
781  aagactttaa agatcttgca agatgcttat gctaagaaat ataatgctga aactgtaagt
841  cgttaa (SEQ ID NO:1067)
```

Protein Sequence *Ls*DAT (ACCESSION YP_536555 VERSION YP_536555.1 GI:90962639)

```
  1  MKQVGYYNGT IADLNELKVP ATDRALYFGD GCYDATTFKN NVAFALEDHL DRFYNSCRLL
 61  EIDFPLNRDE LKEKLYAVID ANEVDTGILY WQTSRGSGLR NHIFPEDSQP NLLIFTAPYG
121  LVPFDTEYKL ISREDTRFLH CNIKTLNLLP NVIASQKANE SHCQEVVFHR GDRVTECAHS
181  NILILKDGVL CSPPRDNLIL PGITLKHLLQ LAKENNIPTS EAPFTMDDLR NADEVIVSSS
241  ACLGIRAVEL DGQPVGGKDG KTLKILQDAY AKKYNAETVS R (SEQ ID NO:1068)
```

Example 10—Analysis of DATs

HMS174 *E. coli* containing the DAT nucleic acids having the sequence of SEQ ID NO:865, 867, 869, 871, 873, 875, and 877 in vector pSE420-cHis were obtained and streaked on agar plates containing LB medium with ampicillin. One skilled in the art can synthesize the genes encoding these D-aminotransferases using assembly PCR techniques such as those described in Example 4. Single colonies were used to inoculate 3 mL of LB medium containing ampicillin (100 μg/mL). Five hundred μl of the overnight culture was used to inoculate 50 mL of the same medium in 250 mL baffled flasks. The cells were grown at 30°C to approximately an OD_{600nm} of 0.5. IPTG was added to a final concentration of 1 mM. Cells were induced at 30°C for 4 hours and collected by centrifugation.

Cell extracts were prepared as described in Example 4. Total protein and DAT concentrations were determined as described in Example 4. The DATs all appeared to express well, and most of them showed a high degree of solubility.

A monatin formation assay was done as described in Example 5 except with a DAT concentration of 0.1 mg/mL and the aldolase at a concentration of 10 μg/mL. As a control, 0.1 mg/mL of purified *B. sphaericus* DAT was assayed. After 6 and 22 hours, an aliquot was taken

and formic acid added to a final concentration of 2%, and the samples were frozen. Samples were then thawed, spun and filtered. Samples were analyzed for monatin concentrations using the LC/MS/MS methodology described in Example 3. Under the conditions tested, SEQ ID NO:870, 874 and 878 all appeared to have high activity in the 3-step monatin formation assay. DAT polypeptides having the sequences shown in SEQ ID NOs:866, 872, and 876 also had activity in the monatin formation pathway but not to the same extent as did polypeptides having the sequence shown in SEQ ID NOs:870, 874 and 878 under the conditions tested. Table 14 shows the results for monatin formation (in ppm).

Table 14. Monatin formation assay

DAT polypeptide (SEQ ID NO)	6 hr	22 hr
866	17.4	76
868	nd	nd
870	132	836
872	13.8	50
874	281.6	798
876	2.4	12
878	223.4	576
B. sphaericus DAT	175.6	616

nd, not detected under conditions tested

Further analysis of polypeptides having the sequence of SEQ ID NO:870, 874 and 878 in pET30a

Cultures of *E. coli* BL21 DE3 transformed with pET30a plasmids containing nucleic acids encoding the above-indicated DATs were grown overnight in 50 mL of Overnight Express II (Solution 1-6, Novagen) at 30°C. As a positive control, a strain containing the DAT from ATCC #4978 in pET30a was also grown and induced (described in Example 6). Cells were collected at at an OD_{600nm} of 5-10, harvested by centrifugation and frozen at -80°C until further processed.

Cell extracts were prepared as described in the Example 4. Total protein and DAT concentrations were determined as described in Example 4.

A monatin formation assay was done as described in Example 5 except with a DAT polypeptide concentration of 0.5 mg/mL for SEQ ID NO:870 and a concentration of 0.275 mg/mL for each of SEQ ID NO:874 and 878. As a control, DAT4978 and purified *B. sphaericus* DAT were assayed at 0.5 mg/mL concentration. After 0.5, 1, 2, 4, 6.5, 9, 24 and 22 hr, an aliquot was taken and formic acid added to a final concentration of 2% and the samples were frozen. Samples were then thawed, spun and filtered. Samples were analyzed for monatin using the LC/MS/MS methodology described in Example 3. The results are shown in Table 15 (in ppm of monatin formed).

Table 15

DAT polypeptide (SEQ ID NO)	0.5 hr	1 hr	2 hr	4 hr	6.5 hr	9 hr	24 hr
4978 DAT	18	85.6	283.4	673.2	890	1226	2020
870	14.4	71	279.4	736	1340	1680	3362
874	63.8	182.6	415.6	674	888	938	1154
878	97.8	244.4	607	912.2	1068	1174	1356
B. sphaericus	44.6	142.8	375.2	813	1294	1382	2746

All three of the subcloned DATs (encoding polypeptides having the sequence of SEQ ID NO:870, 874, and 878) expressed well in the pET system and yielded soluble protein. The polypeptide having the sequence shown in SEQ ID NO:870 gave the highest amount of expression in the soluble fraction and exhibited high activity that did not appear to diminish over time in comparison to the DAT polypeptides having the sequence of SEQ ID NO:874 and 878.

Comparison between wild type and mutant DAT polypeptides

A mutant polypeptide in which the residue of SEQ ID NO:870 was changed from a T to a N (SEQ ID NO:870 T242N) was constructed as described in Example 4 and expressed and compared to DAT4978, DAT4978 T243N (described in Example 6), *B. sphaericus* and wildtype SEQ ID NO:870.

Cultures of BL21 DE3 in which the wild type and mutant polypeptides having the sequence of SEQ ID NO:870, 870 T242N, DAT4978 and DAT4978 T243N were expressed from the pET30a vector, were grown in 50 mL of Overnight Express (Novagen) in a 250 mL baffled flask overnight at 30°C and 250 rpm. The cells were collected by centrifugation when they reached an optical density at 600 nm of over 10. Cell extracts were prepared as described in Example 4, and total protein and DAT concentrations were determined as described in Example 4. All of the DATs tested were highly expressed and soluble, all near 30% as determined using the Experion software. The polypeptide having the sequence of SEQ ID NO:870 T242N had the highest expression, which was predicted to be 36.3 % of the total soluble protein.

A monatin formation assay was done as described in Example 5 at a DAT polypeptide concentration of 0.5 mg/mL. As a control, 0.5 mg/mL of purified *B. sphaericus* DAT was assayed. After 0.5, 1 2, 4, 6.5, 9 and 23.25 hr, an aliquot was taken, formic acid added to the aliquot to a final concentration of 2% and the samples frozen. Samples were then thawed, spun and filtered. Samples were analyzed for monatin, tryptophan, alanine and 4-hydroxy-4-methyl glutamic acid (HMG) as described in Example 3.

In the last time point, an additional aliquot was taken to determine %R,R monatin by the FDAA-derivatization method described in Example 3.

Monatin formation numbers (ppm) are presented in Table 16 below. The percent R,R is given in the right-hand column, for the 23.25 hr timepoint.

Table 16

DAT polypeptide (SEQ ID NO)	0.5 hr	1 hr	2 hr	4 hr	6.5 hr	9 h r	23.25 hr	% R,R
Wild type DAT 4978	11	57	216	472	694	942	1616	95.0
4978 T243N	74	237	542.6	1106	1396	1784	2202	99.0
870	15.6	74.4	269.6	702	1250	1522	2788	97.8
870 T242N	49.4	194	655.2	1496	2212	2666	3670	99.5
B.sphaericus	40.6	144	372	800	1090	1458	2434	97.2

The activity of the T242N mutant of the SEQ ID NO:870 polypeptide was very high, and was better than the positive controls and higher than the wildtype form of DAT polypeptides. The percentage of R,R monatin formed by this mutant was also higher than any of the other benchmark enzymes. The analysis of the amount of HMG (a by-product) formed is qualitative, but it appears that, at the 9 hour and 23.25 hour timepoints, similar amounts of HMG were formed by DAT 4978 T243N polypeptides and SEQ ID NO:870 T242N polypeptides.

The DAT polypeptide having the sequence shown in SEQ ID NO:870 is a novel protein, exhibiting 76% sequence identity to the closest known D-aminotransferase (*Bacillus* YM-1 D-aminotransferase) and 69% amino acid sequence identity to the *B. sphaericus* DAT described in Example 6. Figure 2 shows an alignment of this novel enzyme with other published DATS, and one can see the residues that make this enzyme unique and may account for its superior activity.

The highly active DAT polypeptide having the sequence shown in SEQ ID NO:910 (more similar to *B. sphaericus* type DATs; see Example 12) is also shown in the alignment. As an example of the uniqueness of the SEQ ID NO:870 polypeptide, in the region surrounding amino acid residue 54-55 (*B. sphaericus* numbering) in the alignment of Figure 2, it is clear that the *Bacillus*-like DATs have a high degree of conservation whereas SEQ ID NO:870 has the residues EC rather than AS. As another example, in the highly conserved region surrounding residue 135 of the alignment shown in Figure 2, the SEQ ID NO:870 polypeptide has a more hydrophilic residue (T) versus predominantly valine residues. The core sequence that represents the SEQ ID NO:870 enzyme, but excludes previously known broad specificity D-aminotransferases and highly related homologs, is shown as Consensus Sequences A and B. It is expected that any polypeptide containing one of these consensus sequences would exhibit DAT activity and be active in monatin formation pathway steps.

Consensus Sequence A

Y.*LWND.*IV.*EDRGYQFGDG.*YEV.*KVY.*G.*FT.*EH.*DR.*YECAEKI.*PYTK.*H.*L LH.*L.*E.*N.*TG.*YFQ.*TRGVA.*RVHNFPAGN.*Q.*V.*SGT.*K.*F.*R.*N.*KGVKAT.* TED.*RWLRCDIKSLNLLGAVLAKQEAIEKGCYEA.*LHR.*G.*TE.*SS.*N.*GIK.*GTLYT HPA.*N.*ILRGITR.*V.*TCAKEIE.*PV.*Q.*T.*K.*LEMDE.*V.*S.*SE.*TP.*I.*DG.*KI.*N G.*G.*WTR.*LQ.*F.*K.*P (SEQ ID NO:1069).

Consensus Sequence B

Y[ST]LWND[QK]IV.[DE].{2}[VI].[IV].{2}EDRGYQFGDG[IV]YEV[IV]KVY[ND]G.[ML]F T.{2}EH[IV]DR.YECAEKI[RK][LIV].[IV]PYTK.{3}H[QK]LLH.L[VI]E.N.[LV].TG[HN][IVL]YFQ[IV]TRGVA.RVHNFPAGN[VI]Q.V[LI]SGT.K.F.R.{3}N.[EQ]KGVKAT.TED[IV]RWL RCDIKSLNLLGAVLAKQEAIEKGCYEA[IV]LHR.G.[VI]TE.SS.N[VI][FY]GIK[DN]GTLYT HPA[ND]N.ILRGITR.V[IV][LI]TCAKEIE[LMI]PV.[EQ]Q.{2}T.K.{2}LEMDE[LIVM].V[ST] S.[TS]SE[IV]TP[VI]I[DE][IVL]DG.KI.NG.{2}G[ED]WTR[KQ]LQ.{2}F.{2}K[IL]P. (SEQ ID NO:1070).

Similar to PERL regular expression convention language, ".*" indicates that any number of amino acid residues may be present from any of the 20 proteinogenic amino acids; [ ] indicates that any one of the amino acids in the brackets can be present; ".{#}" indicates that any of the 20 proteinogenic amino acids can be present as long as the number of residues matches the number {#} indicated in the brackets.

With respect to the use of ".*" in Consensus sequence A, the number of amino acids at any of the ".*" positions can vary, for example, from 0 to about 20 residues (see, for example, Consensus sequence B (SEQ ID NO:1070)) or from about 20 residues up to about 100 residues, or the number of amino acids can be much larger, for example, up to 1000 or more residues. Without limitation, an insertion at one or more of the ".*" positions can correspond to, for example, a domain such as (but not limited to) a chitinase binding domain (e.g., from *Pyrococcus furiosus* (Accession No. 2CZN_A) or *P. burkholderia* (Accession No. YP_331531) or a cellulose binding domain (e.g., from *Cellulomonas fimi* (Accession No. 1EXH_A) or *Clostridium stercorarium* (Accession No. 1UY1_A). In some embodiments (without limitation), five or less of the positions designated ".*" each contain an insertion of, for example, greater than about 20 residues (e.g., greater than about 100 residues). In other embodiments (without limitation), five or more of the positions designated ".*" each contains an insertion of less than about 100 residues (e.g., less than about 20 residues, e.g., 3, 5, 10, 20, 25, 30, 40, 50, 60, 70, 75, 80, 90 or 95 residues)). The activity of a polypeptide having a sequence that corresponds to one

or more of the consensus sequences disclosed herein and containing any number of residues inserted at one or more of the ".*" positions can be evaluated using methods that are described herein.

Non-limiting representative polypeptides that contain the consensus sequence shown in SEQ ID NO:1069 include the polypeptide having the sequence shown in SEQ ID NO:870 and Consensus sequence B (SEQ ID NO:1070).

Comparison between SEQ ID NO:870, 870 T242N, DAT 4978 and DAT 4978 T243N, tagged and untagged

E. coli BL21 DE3 cells expressing the polypeptides having the sequence of SEQ ID NO:870, 870 T242N, DAT 4978 and DAT 4978 T243N were grown in 50 mL of Overnight Express (Novagen) in a 250 mL baffled flask overnight at 30°C and 250 rpm. The cells were collected by centrifugation when the optical density at 600 nm was greater than 10. Cell extracts were prepared as described in Example 4. Total protein and DAT concentrations were determined as described in Example 4.

E. coli BL21 DE3 cultures expressing C-terminal tagged SEQ ID NO:870, 870 T242N, DAT 4978 and DAT 4978 T243N polypeptides were grown in 200 mL of Overnight Express (Novagen) in a 1000 mL baffled flask overnight at 30°C and 250 rpm. Two cultures of each clone were grown. The cells were pooled and collected by centrifugation when at an optical density at 600 nm of over 10. Cell extracts were created by the addition of 50 mL of Bug Buster Primary Amine Free (Novagen) with 50 μl of Benzonase Nuclease (Novagen), 0.75 μl of rLysozyme (Novagen), and 250 μl of Protease Inhibitor Cocktail II (Calbiochem). The cells were incubated for 15 minutes at room temperature with gentle rocking. The extracts were centrifuged at 45,000 xg for 10 minutes.

The His-tagged proteins were purified as described in the methods section using GE Healthcare (Piscataway, NJ) Chelating Sepharose™ Fast Flow resin. The purified protein was desalted using a PD10 column into 100 mM potassium phosphate, pH 7.8 (for DAT 4978 and DAT 4978 T243N polypeptides) or 100 mM EPPS pH 8.2 (for SEQ ID NO:870 and 870 T242N polypeptides), both buffers contained 50 μM PLP.

An R,R monatin formation assay was performed as described in Example 5 with a DAT concentration of 0.5 mg/mL. Aliquots were taken at 2.25, 4.5, 9 and 24 hours, pH adjusted with formic acid, and frozen. An extra aliquot was taken at the final time point without formic acid addition for determination of stereoisomeric distribution using the FDAA derivatization method described in Example 3. The samples were thawed and centrifuged for 5 minutes and the supernatant filtered with a 0.2 μm nylon membrane filter. Samples were submitted for monatin analysis using the LC/MS/MS method described in Example 3. The results are shown in Table

17, in ppm monatin formed. The far right column is the % R,R monatin formed at the end of the experiment.

Table 17

DAT polypeptide (SEQ ID NO)	2.25 hr	4.5 hr	9 hr	24 hr	% R,R
DAT 4978	330	676	1470	2384	92.3
DAT 4978 T243N	1392	2856	4068	3688	98.2
870	395	952	1896	2998	97.8
870 T242N	1416	2936	3868	3976	99.3
DAT 4978 6xHis tagged	362	887	1664	2818	96.5
DAT 4978 T243N 6xHis tagged	1364	2298	3464	4440	98.9
870 6xHis tagged	228	688	1508	3138	98.1
870 T242N 6xHis tagged	746	2020	3962	4552	99.5

The overall activity and stereospecificity of the C-terminally tagged and untagged enzymes are very similar. In addition, it is expected that the presence of activity in a polypeptide encoded from a subcloned nucleic acid is predictive of the presence of activity in the corresponding polypeptide encoded from the full-length or wild type nucleic acid.

Example 11—Analysis of DATs

E. coli HMS174 containing DAT nucleic acids in the pSE420-cHis vector encoding the polypeptides having the sequence of SEQ ID NO:880, 882, and 884 were streaked onto agar plates containing LB medium with ampicillin. One skilled in the art can synthesize the genes encoding these D-aminotransferases using assembly PCR techniques such as those described in Example 4. Single colonies were used to innoculate 3 mL of LB medium containing ampicillin (100 μg/mL). Five hundred μl was used to inoculate 50 mL of the same medium in a 250 baffled flask. The cells were grown at 30°C to approximately an OD_{600nm} of 0.4, and IPTG was added to a final concentration of 1 mM. Cells were grown at 30°C for 4 hours and collected by centrifugation.

Cell extracts were prepared as described in the Example 4. Total protein and DAT polypeptide concentrations were determined as described in Example 4. SEQ ID NO:882 and 884 expressed well and were present at high levels in the soluble fraction.

An R,R monatin formation assay was performed as described in Example 5 using approximately 0.5 mg/mL of each DAT polypeptide (except that 0.35 mg/mL of the SEQ ID NO:880 polypeptide was utilized). After 2, 8, and 23 hours, an aliquot was taken, formic acid was added to a final concentratin of 2%, and the samples were frozen. Samples were then thawed, spun and filtered. Samples were analyzed for monatin using the LC/MS/MS methodology described in Example 3. Results are shown in Table 18.

At the last time point, an extra aliquot was taken (without pH adjustment) to determine the stereoisomeric distribution of the monatin produced using the FDAA derivatization methodology described in Example 3. The percentage of R,R produced is shown in the right hand column of Table 18 below, the balance is predominantly S,R monatin.

Table 18

Polypeptide (SEQ ID NO)	monatin ppm (2 hr)	monatin ppm (8 hr)	monatin ppm (23 hr)	% R,R (23 hr)
880	31.6	140	176	97.5
882	31.6	872	2790	99.3
884	79.4	644	1610	100
B. sphaericus	337	1518	2538	96.7

Polypeptides having the sequence shown in SEQ ID NO:882 and 884 exhibited good activity in the monatin formation reactions from D-tryptophan.

The stereopurity of the monatin produced was higher when using these DATs as compared to the *B. sphaericus* control enzyme. The DAT nucleic acids encoding DAT polypeptides having the sequence of SEQ ID NO:882 and 884 were subcloned into pET30a vectors as described in Example 4.

Analysis of DAT polypeptides having the sequence of SEQ ID NO:882 and 884 expressed from the pET30a vector

Cultures of *E. coli* BL21 DE3 containing nucleic acids encoding DAT polypeptides having the sequence of SEQ ID NO:882 and 884 in the pET30a vector were grown in 50 mL of Overnight Express (Novagen) in a 250 mL baffled flask overnight at 30°C and 250 rpm. The cells were collected by centrifugation when the optical density at 600 nm was greater than 10.

Cell extracts were prepared as described in Example 4. Total protein and DAT polypeptide concentrations were determined as described. Total and soluble protein samples were analyzed using a 4 – 15% gradient acrylamide gel as well as by the Experion system. Expression was predicted to be approximately 30% by the Experion software. Visible bands were seen for both the total protein and soluble protein (cell-free extract) fractions.

A monatin formation assay was performed as described in Example 5 with both 0.5 and 2 mg/mL DAT polypeptide concentrations. Purified B. *sphaericus* DAT was used as a control. After 2, 4.5, 9, 24, 36 and 48 hrs, an aliquot was taken, formic acid was added to a final concentration of 2%, and the samples were frozen. Samples were then thawed, spun and filtered. Samples were analyzed for monatin using the LC/MS/MS methodology described in Example 3. The samples were qualitatively analyzed for HMG levels. Additional aliquots were taken

(without pH adjustment) for stereoisomeric analysis using the FDAA derivatization methodology described in Example 3. The results are shown in Tables 19 and 20.

Table 19

DAT polypeptide (SEQ ID NO)	Monatin (ppm) 2 hrs	Monatin (ppm) 4.5 hrs	Monatin (ppm) 9 hrs	Monatin (ppm) 24 hrs	Monatin (ppm) 36 hrs	Monatin (ppm) 48 hrs
882 (0.5 mg/mL)	61	274	780	1802	2172	2170
882 (2 mg/mL)	985	2452	3232	3128	3082	3158
884 (0.5 mg/mL)	149	362	656	1394	1756	2158
884 (2 mg/mL)	811	1628	2466	2988	3178	2864
B. sphaericus (0.5 mg/mL)	362	860	1268	2362	2532	2804
B. sphaericus (2 mg/mL)	1335	2344	3154	3866	3842	4008

Table 20. Stereopurities of monatin produced at selected timepoints

DAT polypeptide (SEQ ID NO)	24 hrs (% R,R)	48 hrs (% R,R)
882 (2mg/mL)	95.4	94.1
884 (2mg/mL)	99.6	99.4
Bs DAT (2mg/mL)	95.8	93.3

Polypeptides having the sequence shown in SEQ ID NO:882 and 884 exhibited good monatin formation activity and stereospecifiity, and appeared to produce less HMG than the *B. sphaericus* control during the initial timepoints. Polypeptides having the sequence of SEQ ID NO:882 exhibited similiar initial monatin formation rates but appeared to have plateaued in this experiment at a lower monatin titer.

Example 12—Analysis of DATs in pSE420-cHis, and of a DAT in pET30a

The open reading frames encoding DAT polypeptides having the sequence of SEQ ID NO:898, 900, 902, 904, 906, 910, and 896 were evaluated. One of ordinary skill in the art can synthesize the genes encoding these D-aminotransferases using assembly PCR techniques such as those described in Example 4.

A culture of *E. coli* BL21 DE3 containing a nucleic acid encoding a DAT polypeptide having the sequence shown in SEQ ID NO:896 in the pET30a vector (subcloned as described in Example 4) was grown in 50 mL of Overnight Express (Novagen) in a 250 mL baffled flask

overnight at 30°C and 250 rpm. The cells were collected by centrifugation when the optical density at 600 nm was greater than 10.

Top10 (Invitrogen, Carlsbad, CA) *E. coli* cells were transformed with the pSE420-cHis plasmid containing the DAT nucleic acids having the sequence shown in SEQ ID NO:897, 899, 901, 903, 905, and 909 and plated on LB medium containing ampicillin (100 μg/mL). Five hundred μl of an overnight culture was used to inoculate 50 mL of the same medium in a 250 baffled flask. The cells were grown at 30°C to an OD_{600nm} of approximately 0.4 and IPTG was added to a final concentration of 1 mM. Cells were grown at 30°C for 4 hours and collected by centrifugation.

Cell extracts were prepared as described in Example 4. Soluble protein and estimated DAT concentrations were determined as described in Example 4.

An R,R monatin formation assay was performed as described in Example 5 with DAT polypeptide concentrations of 0.5 mg/mL, except that 0.3 mg/mL of the polypeptide having the sequence of SEQ ID NO:896 was used; 0.06 mg/mL of the polypeptide having the sequence of SEQ ID NO:898 was used; 0.4 mg/mL of the polypeptide having the sequence shown in SEQ ID NO:900 was used; 0.1 mg/mL of the polypeptide having the sequence of SEQ ID NO:902 was used; and 0.12 mg/mL of the polypeptide having the sequence of SEQ ID NO:904 was used. As positive controls, SEQ ID NO:870, 870 T242N, and purified *B. sphaericus* were tested at DAT polypeptide concentrations of 0.5 mg/mL. After 2, 6, and 24 hours, an aliquot was taken, formic acid was added to a final concentration of 2% and the samples were frozen. Samples were then thawed, spun and filtered. Samples were analyzed for monatin using the LC/MS/MS methodology described in Example 3. Additional aliquots were taken for stereoisomeric distribution analysis and were not treated with formic acid. The results for the 24 hour time point are shown in Table 21. The DAT nucleic acid encoding the DAT polypeptide having the sequence shown in SEQ ID NO:908 was not subcloned and could not be assayed.

Table 21

Polypeptide (SEQ ID NO)	monatin 2 hrs (ppm)	monatin 6 hrs (ppm)	monatin 24 hrs (ppm)	24 hr %R,R
B. sphaericus	261	1203	2604	95.6
870	193	1067	2490	96.8
870 T242N	813	2230	3380	98.8
896	30	127	286	99.8
898	nd	3	15	95.7
900	4	16	56	92.9
902	144	411	1209	96.7
904	nd	1	4	92.3
906	14	18	25	98

910	487	1154	2770	94.5

nd=not detectable under conditions tested

DAT polypeptides having the sequence shown in SEQ ID NO:910 and 902 had high levels of activity for the monatin formation reactions, and produced fairly high levels of R,R monatin. Results indicated that the DAT polypeptide having the sequence shown in SEQ ID NO:870 exhibited comparable activity to that of the wildtype polypeptide having the sequence shown in SEQ ID NO:910 under the conditions tested; however, the T242N mutation in the SEQ ID NO:870 polypeptide makes a large improvement in activity and stereospecificity of the enzyme.

Example 13—Analysis of DATs

Plasmids (pSE420-cHis) containing the nucleic acid sequences encoding SEQ ID NO:912, 914, 916, 918, 920, 922, 924, and 926 DATs were obtained. One skilled in the art could clone the genes using any number of gene assembly protocols such as the one described in Example 4.

E. coli Top10 (Invitrogen) cells were transformed with the pSE420-cHis plasmids containing DAT polypeptides having the sequence of SEQ ID NO:912, 914, 916, 918, 920, 922, 924, and 926 and plated on LB medium containing ampicillin (100 μg/mL). Five hundred μl of the overnight culture was used to inoculate 50 mL of the same medium into 250 mL baffled flasks. Cultures were grown at 30°C to an OD_{600nm} of approximately 0.4. IPTG was added to a final concentration of 1 mM. Cells were grown at 30°C for 4 hours and collected by centrifugation.

Cell extracts were prepared as described in Example 4. Total soluble protein and DAT protein concentrations were determined as described in Example 4. Most of the DAT polypeptides that were expressed were soluble except for the SEQ ID NO:916 polypeptide, which was only partially soluble.

An R,R monatin formation assay was performed as described in Example 5, with a DAT polypeptide concentration targeted to about 0.25 mg/mL; except that 0.1 mg/mL of the SEQ ID NO:922 polypeptide was used and 0.2 mg/mL of the SEQ ID NO:926 polypeptide was used. As a positive control, purified *B. sphaericus* DAT was tested at a 0.25 mg/mL concentration. After 2, 8 and 24 hours, an aliquot was taken and formic acid was added to a final concentration of 2%, and the samples were frozen. Samples were then thawed, spun and filtered. Samples were analyzed for monatin using the LC/MS/MS methodology described in Example 3. The results are shown in Table 22.

Table 22

Polypeptides (SEQ ID NO)	monatin (ppm) 2 hours	monatin (ppm) 8 hours	monatin (ppm) 24 hours
B. sphaericus	179	774	1482
912	0.2	2	2
914	4	22	62
916	0.6	2	6
918	158.8	402	496
920	5	40	96
922	0.2	2	2
924	nd	nd	nd
926	1.2	12	30

nd=not detected under conditions assayed

DAT nucleic acids having the sequence shown in SEQ ID NO:169, 171, 167, 173 and 175 (encoding DAT polypeptides having the sequence shown in SEQ ID NO:170, 172, 168, 174 and 176) were obtained as PCR products and were subcloned in pET30a as described in Example 4. One of ordinary skill in the art could reconstruct the genes using any number of gene assembly methods such as the one described in Example 4.

E. coli BL21 DE3 cells containing DAT nucleic acids having the sequence of SEQ ID NO:169, 171, 167, 173 and 175 in the pET30a vector were grown in 50 mL of Overnight Express (Novagen) in a 250 mL baffled flask, overnight at 30°C and 250 rpm. The cells were collected by centrifugation when the optical density at 600 nm was greater than 10.

Cell extracts were prepared as described in the Example 4. Total soluble protein and DAT protein concentrations were determined as described in Example 4. The polypeptide having the sequence of SEQ ID NO:170 (encoded by the DAT nucleic acid having the sequence of SEQ ID NO:169) did not appear to be soluble, which may have impeded activity assays.

An R,R monatin formation assay was performed as described in Example 5 using a DAT polypeptide concentration of 0.5 mg/mL, except that 0.25 mg/mL of the SEQ ID NO:170 polypeptide was used. As a positive control, purified *B. sphaericus* DAT was tested at a 0.5 mg/mL concentration. After 2, 8 and 24 hours, an aliquot was taken, formic acid was added to a final concentration of 2%, and the samples were frozen. Samples were then thawed, spun and filtered, and analyzed for monatin using the LC/MS/MS methodology described in Example 3. Results are shown in Table 23.

Table 23

Polypeptides (SEQ ID NO)	monatin (ppm) 2 hr	monatin (ppm) 8 hr	monatin (ppm) 24 hr
B. sphaericus	456	1502	2970
170	2	8	14
172	5	20	60
168	15	68	186
174	1	4	8
176	451	1508	2744

Samples (without pH adjustment) were analyzed to determine % R,R using the FDAA derivatization protocol described in Example 3. The monatin produced by DAT polypeptide having the sequence shown in SEQ ID NO:176 was 99.6% R,R after 24 hrs compared to that produced by *B. sphaericus,* which was 95.2% R,R at the same timepoint. The activity and stereopurity resulting from the DAT polypeptide having the sequence of SEQ ID NO:176 were both quite high, and the corresponding nucleic acid was subcloned as a C-terminal tagged protein as described in Example 4 for more quantitative studies.

Characterization of SEQ ID NO:176 C-His-tagged protein

The nucleic acid having SEQ ID NO:175, which encodes the polypeptide having the sequence of SEQ ID NO:176, was cloned into pET30a without a stop codon so that it could be expressed as a fusion protein with a 6xHis-tag on the C-terminus. The protein was purified using the His-bind resin described in Example 4. When the fusion protein was eluted from the PD-10 desalting column, a yellow precipitate formed in the solution. A yellow residue was also observed on the column. The yellow color usually is indicative of the presence of a PLP-binding protein. In an effort to prevent the precipitation of the PLP-binding protein at the desalting step, different buffers (100 mM phosphate and 100 mM EPPS with or without 10% glycerol) at two pH values (7.8 and 8.2) were utilized. None of the buffers tried appeared to completely prevent the precipitation.

The monatin assay was performed using a well-mixed heterogeneous protein solution and a DAT polypeptide concentration of 0.5 mg/mL. The results are shown in Table 24. The purified SEQ ID NO:176 DAT polypeptide (C-tagged) showed comparable activity to the positive control DAT polypeptide from *B. sphaericus*; however, the activity appeared to be lower than the activity exhibited by the mutant polypeptides having the sequence of SEQ ID NO:870 T242N or SEQ ID NO:870 T242N.

Table 24. Monatin Production (ppm)

Polypeptide (SEQ ID NO)	2 hr	4 hr	8 hr	24 hr
B. sphaericus	262	676	1044	2150
870	332	678	1346	2826
870 T242N	942	1938	2834	4004
176	208	392	732	1806

Example 14—Evaluation of DATs

The open reading frames encoding 29 DATs were obtained as PCR products. It is noted that one of ordinary skill in the art can synthesize the genes encoding the DATs using assembly techniques such as those described in Example 4. The DAT nucleic acids were subcloned into the pET30a vector and expressed as untagged proteins as described in Example 4. The desalted cell-free extracts (prepared as described in Example 4) were used in monatin formation assays. A DAT polypeptide concentration of 0.5 mg/mL was used for the monatin assay except for the following polypeptides (amounts used in parentheses): the SEQ ID NO:156 polypeptide (0.4 mg/mL), the SEQ ID NO:182 polypeptide (0.45 mg/mL), the SEQ ID NO:240 polypeptide (0.47 mg/mL), and the SEQ ID NO:204 polypeptide (0.42 mg/mL).

Most of the DAT polypeptides showed undectectable to low monatin production under the conditions assayed as compared to positive control enzymes. Most of the expressed DAT polypeptides were soluble as determined by the Experion; however, the polypeptides having SEQ ID NO:204 and 240 were expressed at very low levels and may not have been very soluble. On the other hand, the polypeptide having SEQ ID NO:220 was predicted to be 68% of the total soluble protein as judged by the Experion software.

The monatin production results are shown in Table 25 and 26. At 24 h, the DAT polypeptide having SEQ ID NO:156 and 214 produced 40-50% of monatin as compared to the positive control enzyme, the DAT from *B. sphaericus*. The most active DAT polypeptide was the SEQ ID NO:220 polypeptide. Approximately 4 h after the reaction was started, the monatin concentration reached a maximum. It is expected that the mature protein of SEQ ID NO:156 (without the predicted leader sequence) is the active component of the DAT polypeptide and, therefore, the protein can be produced recombinantly with the leader sequence absent.

Table 25. Monatin Formed (ppm)

Polypeptide (SEQ ID NO)	2 hr	4 hr	8 hr	24 hr
178	11	24	62	194
180	nd	nd	nd	nd
154	52	103	166	178
182	nd	nd	nd	nd

218	1.6	2.8	274	12
188	2.4	5.2	10	22
190	3.8	9.4	22	42
208	1	1.2	nd	nd
220	2418	3563	3812	3882
196	1	1.8	nd	8
156	64.8	156	296	796
B. sphaericus	422	791	1302	2124

nd=not detectable under conditions assayed

Table 26. Monatin Formed (ppm)

Polypeptide (SEQ ID NO)	2	4	8	24
166	nd	nd	1	nd
216	69	91	109	134
200	nd	nd	1	1
198	nd	nd	nd	nd
210	1.6	3.8	6.2	15.2
202	3.4	6.2	12.2	29.8
222	3.6	7	12.8	25.8
236	nd	nd	nd	nd
204	nd	nd	nd	3.6
238	10.4	21.8	46.4	115.6
240	3	6	12.4	32.2
224	39.8	85	171.8	268.8
226	2.6	5.8	12.4	30.6
228	4.2	9.8	21.4	66.8
230	9.2	21.8	42.2	94.4
232	3.6	9.4	21.6	57
246	nd	nd	nd	nd
214	160	327	694	1346
B. sphaericus	393	986	1624	2597

nd=not detectable under conditions assayed

The high activity of the SEQ ID NO:220 polypeptide was confirmed in another monatin formation assay where the SEQ ID NO:220 polypeptide was compared to the SEQ ID NO:870, 870 T242N, and *B. sphaericus* DAT polypeptides. A DAT polypeptide concentration of 0.1 mg/mL and 0.5 mg was used for each of the DAT polypeptides assayed. The results are shown in Table 27. Due to the high degree of activity of the DAT polypeptides assayed, the monatin samples had to be diluted 100-fold to be within the quantitative range of the instruments used (as opposed to a typical 10- or 20-fold dilution).

Table 27. Monatin Formed (ppm)

DAT Polypeptide (SEQ ID NO)	2 hr	4 hr	8 hr	24 hr
B. sphaericus (0.1 mg/mL)	74	170	309	728

B. sphaericus (0.5 mg/mL)	510	921	1068	2704
870 (0.1 mg/mL)	28	81	179	706
870 (0.5 mg/mL)	399	847	1466	2916
870 T242N (0.1 mg/mL)	93.2	245.8	582.4	1270
870 T242N (0.5 mg/mL)	1158.8	2026	3202	4126
220 (0.1 mg/mL)	965.8	1512	2330	3788
220 (0.5 mg/mL)	2950	4302	4618	4488

The percentage of R,R formed by the DAT polypeptide having SEQ ID NO:220 in the above experiments was determined using the FDAA derivatization methodology described in Example 3. The DAT polypeptide having the sequence of SEQ ID NO:220 is highly stereospecific, producing 99.3% R,R monatin at 24 hours as compared to 92.9% R,R for *B. sphaericus*. In another assay, the SEQ ID NO:220 polypeptide produced 99.8% R,R-monatin.

The DAT polypeptide having the sequence of SEQ ID NO:220 is a novel protein that is 62% identical at the protein level to the *C. beijerinckii* DAT polypeptide shown in Example 9. The SEQ ID NO:220 polypeptide has 86%-90% primary sequence homology to other highly active DAT polypeptides (e.g., those having the sequence shown in SEQ ID NO:892 and 894 (Example 8), 946 (Example 7) and 176 (Example 13)). These highly active and novel DAT polypeptides were uncharacterized prior to this work, and these enzymes exhibited higher activity and stereospecificity for R,R monatin production reactions than any of the published *Bacillus*-like D-aminotransferases. Figure 3 shows an alignment of these related D-aminotransferases and the consensus sequence motifs they have in common are described below.

Consensus Sequence C

M.*GYYNG.*P.*DR.*FGDG.*YDAT.*N.*FAL.*H.*RF.*NS.*LL.*I.*K.*YWQ.*RG.*G.*R.*H.*F.*N.*I.*P.*KLI.*DTRF.*HCNIKTLNL.*P.*VIA.*Q.*E.*C.*E.*VFHRG.*VTECAHSN.*I.*NLIL.*G.*HL.*P.*E.*F.*L.*ADE.*V.*SS.*DG.*GGK.*K.*Q.*T (SEQ ID NO:1071)

Consensus Sequence D

M.{3}GYYNG.{10}P.{2}DR.{3}FGDG.YDAT.{3}N.{3}FAL.{2}H.{2}RF.NS.{2}LL.I.{9}K.{17}YWQ.{2}RG.G.R.H.F.{5,7}N.{2}I.{3}P.{10}KLI.{3}DTRF.HCNIKTLNL.P.VIA.Q.{3}E.{2}C.E.VFHRG.{2}VTECAHSN.{2}I.{11}DNLIL.G.{4}HL.{9}P.{2}E.{2}F.{4}L.{2}ADE.{2}V.SS.{10}DG.{3}GGK.{5}K.{2}Q.{10}T (SEQ ID NO:1072)

Consensus Sequence E

M.*[LV]GYYNG.*[LI].*[ML].*[VI]P.*DR.*[YF]FGDG.*YDAT.*N.*FAL[DE][ED]H[IL][DE]RF.*NS.*LL.*I.*[KR].*[EQ][LMV]K[KE].*[MV].*[DE].*[VL]YWQ.*[TS]RG[TS]G.*R[NS]

H.*F.*N[LI].*I.*P.*[IVL].*[KE].*KLI[TS].*[ED]DTRF.*HCNIKTLNL[IL]P[NS]VIA[SA]Q[RK].*E.*C.*E.*VFHRG[ED].*VTECAHSN[VI].*I[IL][KR][ND].*[TS].*DNLIL.*G.*HL[LI][QK].*[IV]P.*E.*F[TS][LM].*[ED]L.*ADE[VI][LI]V[ST]SS.*[LMI].*[IL]DG.*GGK.*[LVI]K.*[IL]Q.*[EK][FY].*T (SEQ ID NO:1073)

Similar to PERL regular expression convention language (perldoc.perl.org), ".*" indicates that any number of amino acid residues may be present from any of the 20 proteinogenic amino acids; [ ] indicates that any one of the amino acids in the brackets can be present; ".{#}" indicates that any of the 20 proteinogenic amino acids can be present as long as the number of residues matches the number (#) indicated in the brackets.

With respect to the use of ".*" in Consensus sequence C, the number of amino acids at any of the ".*" positions can vary, for example, from about 0 to about 20 residues (see, for example, Consensus sequences D (SEQ ID NO:1072) and E (SEQ ID NO:1073)) or from about 20 residues up to about 100 residues, or the number of amino acids can be much larger, for example, up to 1000 or more residues. Without limitation, an insertion at one or more of the ".*" positions can correspond to, for example, a domain such as (but not limited to) a chitinase binding domain (e.g., from *Pyrococcus furiosus* (Accession No. 2CZN_A) or *P. burkholderia* (Accession No. YP_331531) or a cellulose binding domain (e.g., from *Cellulomonas fimi* (Accession No. 1EXH_A) or *Clostridium stercorarium* (Accession No. 1UY1_A). In some embodiments (without limitation), five or less of the positions designated ".*" each contain an insertion of, for example, greater than about 20 residues (e.g., greater than about 100 residues). In other embodiments (without limitation), five or more of the positions designated ".*" each contains an insertion of less than about 100 residues (e.g., less than about 20 residues, e.g., 3, 5, 10, 20, 25, 30, 40, 50, 60, 70, 75, 80, 90, or 95 residues). The activity of a polypeptide having a sequence that corresponds to one or more of the consensus sequences disclosed herein and containing any number of residues inserted at one or more of the ".*" positions can be evaluated using methods that are described herein.

Non-limiting representative polypeptide that contain the consensus sequence shown in SEQ ID NO:1071 include SEQ ID NO:220, 892, 894, 176 and 946 and Consensus sequences D (SEQ ID NO:1072) and E (SEQ ID NO:1073)). It is expected that any D-aminotransferase exhibiting any of consensus sequences C, D, or E would be active in monatin formation pathway steps.

Characterization of a c-His-tagged polypeptide having the sequence of SEQ ID NO:220

The DAT nucleic acid having the sequence of SEQ ID NO:219 (encoding the polypeptide of SEQ ID NO:220) was cloned into pET30a without a stop codon such that it was expressed as

a fusion protein with a 6xHis-tag on the C-terminus (as described in Example 4). The fusion protein was purified using the His-Bind column (Novagen) described in Example 4. The eluate from the PD-10 desalting column formed a yellow precipitate. Yellow residue was also observed on the column. Monatin assays were done using a well-mixed heterogenous protein solution. Amounts of DAT polypeptide used are indicated in parentheses in the far left column. The notation w/ Trp indicates that the enzyme was incubated with 10 mM D-tryptophan overnight on ice. Results are shown in Table 28.

Table 28. Monatin formed (ppm)

Polypeptide (SEQ ID NO)	2 hr	4 hr	8 hr	24 hr
B. sphaericus (0.5 mg/mL)	382	660	1021	1986
870 T242N (0.1 mg/mL)	69	205	412	1074
870 T242N w/Trp (0.1 mg/mL)	63	163	383	978
870 T242N (0.5 mg/mL)	919	1698	2356	3130
870 T242N w/ Trp (0.5 mg/mL)	772	1519	2294	3023
220 (0.1 mg/mL)	847	1462	2202	3004
220 (0.1 mg/mL)	811	1522	2202	2887
220 w/ Trp (0.1 mg/mL)	537	1080	1590	2401
220 (0.5 mg/mL)	2885	3446	3813	4066
220 w/ Trp (0.5 mg/mL)	1933	3223	3939	3911

The reaction containing 0.1 mg/mL of the SEQ ID NO:220 polypeptide showed a similar monatin formation time course as the reaction containing 0.5 mg/mL of the SEQ ID NO:870 T242N polypeptide. Addition of D-tryptophan (10 mM) to the solution containing the purified protein eliminated the precipitation. Activity loss was observed for the sample in which SEQ ID NO:220 was incubated with D-tryptophan (10 mM) overnight on ice, but no negative effect was observed when the SEQ ID NO:870 T242N polypeptide was treated with D-tryptophan (10 mM). The presence of HMG was also analyzed qualitatively for reactions catalyzed by the SEQ ID NO:220 polypeptide by comparing peak areas. When both DAT polypeptides were utilized at a concentration of 0.5 mg/mL, the reaction catalyzed by the SEQ ID NO:220 polypeptide formed around 40% of the HMG compared to the reaction containing the SEQ ID NO:870 T242N polypeptide. Earlier time points show an even more pronounced difference between the two enzymes.

In an attempt to prevent the protein precipitation during the purification of the SEQ ID NO:220 polypeptide, DTT (5 mM) was included in all the buffers including the Bugbuster reagent, the buffers for His-Bind column and the buffer for PD-10 column. No precipitation was observed after the desalting column, and no negative effect was observed on the activity of the SEQ ID NO:220 polypeptide when DTT was included during purification or added into purified

protein solution at a concentration of 2 mM. Data for monatin formation assays are shown in Table 29. The amount of DAT polypeptide used is indicated in the left column in parentheses. "added DTT" indicates that 2 mM DTT was added to resolubilize the protein after purification; and "purified w/ DTT" indicates that 5 mM DTT was present throughout the purification.

Table 29. Monatin Formed (ppm)

Polypeptide (SEQ ID NO)	2 hr	4 hr	24 hr
B. sphaericus (0.5 mg/mL)	426	965	2638
870 T242N (0.5 mg/mL)	977	1916	4227
220 (0.1 mg/mL)	1214	2163	3964
220 (0.5 mg/mL)	3534	4246	4415
220 added DTT (0.1 mg/mL)	1287	2202	3566
220 added DTT (0.4 mg/mL)	3495	4833	5082
220 purified w/ DTT (0.1 mg/mL)	1204	2169	3997
220 purified w/ DTT (0.5 mg/mL)	3562	4110	4353

The highly desirable properties of the SEQ ID NO:220 polypeptide make it an excellent candidate for further mutagenesis or directed evolution experiments.

Site-directed mutagenesis of the SEQ ID NO:220 polypeptide

A loop region of the DAT polypeptide related to the *Bacillus* DAT polypeptide was identified as being important for the substrate specificity and stereospecificity of the enzymes (Ro et al., 1996, *FEBS Lett,* 398:141-145; Sugio et al., 1995, *Biochemistry* 34:9661-9669; and EP 1 580 268). One key residue in this region is a T at residue 242 (in the DAT polypeptide from ATCC #4978, this position corresponds to a T at residue 243). A T242N mutant of the SEQ ID NO:870 polypeptide showed improvement in both activity and stereospecificity, as did the T243N mutant of DAT 4978 (see Example 10). Primary sequence alignment of the SEQ ID NO:220 polypeptide with the SEQ ID NO:870 polypeptide showed only 35% amino acid sequence identity and 65% homology. The T242 residue in SEQ ID NO:870 aligned with a G240 residue in SEQ ID NO:220, which is followed by a T241 residue. Using Accelrys DS Modeler software for both proteins (with *Bacillus* YM-1 structures as templates), it was difficult to overlap the loop region of the SEQ ID NO:870 polypeptide with the SEQ ID NO:220 polypeptide. Therefore, both amino acids were chosen as targets for site-directed mutagenesis.

A mutant polypeptide designated SEQ ID NO:220 G240N and SEQ ID NO:220 T241N were generated by site-directed mutagenesis of the corresponding nucleic acid (SEQ ID NO:219) as described in Example 4. The two mutant polypeptides were expressed and purified as 6xHis-tagged fusion proteins that were used in the monatin formation assay. Yellow precipitation was observed at the desalting step for both of the mutant SEQ ID NO:220 polypeptides. Results are

shown in Tables 30 and 31 for monatin formation assays. The amount of D-aminotransferase used is indicated in the left-hand column in parentheses. Different preparations of the SEQ ID NO:220 polypeptide were utilized in the assay. "ferm" indicates that the SEQ ID NO:220 polypeptide used was produced in a fermentor as described in Example 15.

Table 30. Monatin Produced (ppm)

Polypeptide (SEQ ID NO)	2 hrs	4 hrs	8 hrs	24 hrs
B. sphaericus (0.5 mg/mL)	440	777	1510	2621
870 T242N (0.5 mg/mL)	961	1913	2793	3904
ferm 220 (0.1 mg/mL)	1396	2379	3217	3770
ferm 220 (0.2 mg/mL)	2301	3277	3789	4328
ferm 220 w/ DTT (0.1 mg/mL)	1434	2384	3109	3730
ferm 220 w/ DTT (0.2 mg/mL)	2423	3568	3859	4755
220 (0.1 mg/mL)	1109	1912	2809	3713
220 T241N (0.1 mg/mL)	554	856	1084	1986

Table 31. Monatin Formed (ppm)

Polypeptide (SEQ ID NO)	2 hr	4 hr	8 hr	24 hr
B. sphaericus (0.5 mg/mL)	634	938	1651	2754
870 T242N (0.5 mg/mL)	1422	1922	3211	3793
ferm 220 (0.1 mg/mL)	1976	2505	3442	4211
ferm 220 (0.2 mg/mL)	3198	3430	4452	4639
220 G240N (0.1 mg/mL)	3	5	14	42
220 G240N (0.2 mg/mL)	9	17	46	94

A very small amount of monatin (95.7% R,R monatin) was formed in the reaction catalyzed by the mutant SEQ ID NO:220 G240N polypeptide. The mutant SEQ ID NO:220 T241N polypeptide lost about 50% of the activity, but still maintained the stereospecificity (99.7% R,R monatin produced). These results, together with the homology modeling and alignments, suggest that, in the region surrounding residues 242-243 (and potentially beyond), the structure of the SEQ ID NO:220 polypeptide is not similar to the structure of the SEQ ID NO:870 polypeptide or the structure of the *Bacillus*-like DAT polypeptide in the literature. Since there is no x-ray crystal structure, random mutagenesis, combinatorial approaches and other directed evolution approaches of the SEQ ID NO:220 polypeptide and related DAT polypeptides are expected to be highly productive in further improving the enzyme's activity.

Example 15—Production of a DAT in a Fermentor

Bacterial growth media components were from Difco, Fisher Scientific, or VWR; other reagents were of analytical grade or the highest grade commercially available. The fermentation was run in a New Brunswick Scientific (Edison, NJ) BioFlo 3000® fermenter. Centrifugation

was carried out using a Beckman (Fullerton, CA) Avanti® J-25I centrifuge with a JLA-16.250 or JA-25.50 rotor.

The DAT nucleic acid encoding the polypeptide having the sequence in SEQ ID NO:220 with a C-terminal His-tag was cloned using *Nde* I / *Xho* I restriction sites into the pMet1a vector described in Example 16. The antibiotic marker (*bla* gene) can further be removed using *Psi* I restriction enzyme digestion, gel purification of the vector band, self- ligation of the vector ends, transformation into the *E. coli* host, and selection on minimal medium plates that do not contain methionine. Typically, Neidhardt's medium with 15 amino acids is used. The cloning sites were *NdeI*/*XhoI* to insert the SEQ ID NO:220 nucleic acid sequence into pMET1a (see Example 16).

The SEQ ID NO:220 DAT polypeptide carrying a C-terminal His-purification tag was produced in a fermentor at the 2.5-L scale, in a fed-batch process that achieves high cell densities and high levels of expression of the desired protein. The protocol and results for growth of *E.coli* strain B834(DE3)::SEQ ID NO:220*cHIS* pMET1 are described as follows: Starting from a fresh culture plate (Neidhardt's +15 amino acids, no methionine), the cells were grown in 5 mL of Neidhardt's medium supplemented with 15 amino acids, at 30°C and 225 rpm for 6-8 h. One mL of the culture was transferred to each of 2 125-mL aliquots of the production medium supplemented with 5 g/L of glucose. The flasks were grown at 30°C and 225 rpm overnight (16-18 h). A fermentor was charged with 2.5 liters of the production medium, containing (per liter): 2.0 g/L $(NH_4)_2SO_4$; 8.0 g/L K_2HPO_4; 2.0 g/L NaCl; 1.0 g/L $Na_3Citrate{\cdot}2H_2O$; 1.0 g/L $MgSO_4{\cdot}7H_2O$; 0.025 g/L $CaCl_2{\cdot}2H_2O$; 0.05 g/L $FeSO_4{\cdot}7H_2O$; 0.4 mL/L Neidhardt micronutrients, and 2.0 g/L glucose. The fermenter was inoculated with 10% v/v of the overnight culture. Three hours after inoculation, an exponential glucose feed was set up using a 60% w/v glucose solution. The feed was supplied at the required rate to support microbial growth at an exponential rate of 0.15 h^{-1}. When the carbon dioxide evolution rate (CER) had reached a value of 100 mmoles/L/h (approximately 21 hours after inoculation; corresponding to a cell biomass of 15-16 g DCW/L), gene expression was induced with a bolus addition of 2 g/L lactose (fed as a 20% solution). The feed was changed from 60% w/v glucose to 50% w/v glucose + 10% w/v lactose while the feed rate was fixed to the rate at time of induction. The "50% w/v glucose + 10% w/v lactose" feed was maintained for 6 hours. At the end of the fermentation, the cells were harvested by centrifugation at 5000-7000 xg for 10 min and frozen as a wet cell paste at –80°C. Cell paste (318 grams) was harvested from 2.8 L of cell broth.

To prepare cell free extract containing the SEQ ID NO:220 polypeptide, 50 g of wet cell paste was suspended in 150 mL of 50 mM EPPS buffer (pH 8.4) containing 50 μM pyridoxal phosphate (PLP) and then disrupted using a Microfluidics homogenizer (Microfluidics, Newton, MA) (3 passes at 18,000 psi), maintaining the temperature of the suspension at less than 15°C.

The cell debris was removed by centrifugation (20,000 xg for 30 minutes). Two mM DTT was added to the clarified cell extract.

To prepare purified SEQ ID NO:220, 2 x 25 mL aliquots of clarified cell extract were loaded each onto a 45 mL Chelating Sepharose™ Fast Flow resin (nickel(II) form) column that had been previously equilibrated with 50 mM EPPS (pH 8.4) containing 0.05 mM PLP and 200 mM sodium chloride. After loading the sample, the column was washed/eluted successively with 3 - 5 volumes of the equilibration buffer, 3 – 5 volumes of the equilibration buffer containing 25 mM imidazole, 3 – 5 volumes of the equilibration buffer containing 100 mM imidazole and 3 – 5 volumes of the equilibration buffer containing 500 mM imidazole. The 500 mM imidazole eluent was concentrated 10X with an Amicon (Billerica, MA) Centricon-70 centrifugal filter device (MWCO 10 kDa). The imidazole and sodium chloride were removed by passage through disposable GE Healthcare PD10 desalting columns previously equilibrated with 50 mM EPPS (pH 8.4) containing 0.05 mM PLP. The protein concentration of the desalted solution was determined using the Pierce BCA assay kit (Rockford, IL). The purity of each fraction and the level of expression in the cell free extract fraction were determined by SDS-PAGE with 4-15% gradient gels. Approximately 450 mg of protein that was ~90% pure was recovered from the 50 mL of clarified cell extract. Two mM DTT was added to 10 mL of the purified protein. The purified protein was dispensed into aliquots (0.5-5 mL) and stored at –80°C.

Bench scale reactions (250 mL) were carried out in 0.7 L Sixfors agitated fermenters (Infors AG, Bottmingen, Switzerland) under a nitrogen headspace. The reaction mix contained 10 mM potassium phosphate, 1 mM $MgCl_2$, 0.05 mM PLP, 200 mM sodium pyruvate and 100 mM D-tryptophan. The reaction mix was adjusted to the appropriate temperature, and adjusted to the appropriate pH with potassium hydroxide. The aldolase described in Example 6 was added as a clarified cell extract at 0.02 mg/mL of target protein. The SEQ ID NO:220 DAT polypeptide was added (either as purified enzyme or as a clarified cell extract) at 0.25 mg/mL of target protein.

The progress of the reactions was followed by measuring monatin concentration using the LC/MS/MS methodology described in Example 3.

Starting with D-tryptophan and under the conditions tested, the pH optimum of the monatin formation reactions using the SEQ ID NO:220 polypeptide was found to be approximately pH 8.0 and the temperature optimum of the monatin formation reactions utilizing the SEQ ID NO:220 polypeptide was found to be approximately 25°C. These reactions have complex dynamics and the optimum reaction conditions for the full monatin production reaction

may not be the same as the optimal conditions for individual reactions catalyzed by the DAT polypeptide.

Example 16—The co-expression of chaperones to increase the soluble expression of a DAT polypeptide

Because the soluble expression of the SEQ ID NO:894 DAT polypeptide was low using the standard expression protocols (either 1 mM IPTG in LB or Novagen Overnight Express Autoinduction System2, see Example 8), co-expression of the SEQ ID NO:894 polypeptide and a variety of commercially available chaperones was examined.

Chaperones:

The TaKaRa Chaperone Set (TAKARA BIO catalog #3340) consists of five different sets of chaperones developed by HSP Research Institute, Inc. They are designed to enable efficient expression of multiple moleculear chaperones known to work in cooperation in the folding process. The set contained the following:

Plasmid	Chaperone	Promoter	Inducer	Resistance Marker
pG-KJE8	*dnaK-dnaJ-grpE; groES-groEL*	*araB; Pzt1*	L-Arabinose; tetracycline	chloramphenicol
pGro7	*groES-groEL*	*araB*	L-Arabinose	chloramphenicol
pKJE7	*dnaK-dnaJ-grpE*	*araB*	L-Arabinose	chloramphenicol
pG-Tf2	*groES-groEL-tig*	*Pzt1*	Tetracycline	chloramphenicol
pTf16	*tig*	*araB*	L-Arabinose	chloramphenicol

Transformation protocol

Chemically competent BL21(DE3) cells (EMD Biosciences/Novagen catalog # 69450) were transformed with 20 ng of one of the TaKaRa chaperone plasmids and 20 ng of SEQ ID NO:893/pET30a (encoding the SEQ ID NO:894 polypeptide; see Example C2 for the plasmid construction) by heat shock for 30 seconds at 42°C. The transformed cells were recovered in 0.5 mL of SOC medium for 1 hr at 37°C and plated on LB plates containing 50 mg/L kanamycin and 25 mg/L chloramphenicol. The plates were incubated overnight at 37°C. Colonies were picked from the overnight plates and used to inoculate 5 mL of 2xYT medium containing 50 mg/L kanamycin and 25 mg/L chloramphenicol. After overnight incubation at 37°C, the plasmids were isolated from the cell pellets using a QUIAprep Spin Miniprep Kit (Qiagen catalog #27104). The plasmids were analyzed by restriction digestion with one-cutter enzymes from New England Biolabs (Beverly, MA) for both the chaperone plasmid and the SEQ ID NO:893/pET30a plasmid following the manufactuere's recommended protocol.

Plasmid	Restriction Enzyme
pG-KJE8	*Xho*I

pGro7	*Xba*I
pKJE7	*Nhe*I
pG-Tf2	*Xho*I
pTf16	*Xba*I

The isolated DNA containing both SEQ ID NO:893/pET30a and pKJE7 was digested with *Nhe*I and *Xba*I.

Expression studies

Flasks of Novagen Overnight Express™ AutoinductionSystem 2 (EMD Biosciences/Novagen catalog #71366) containing solutions 1-6, 50 mg/L kanamycin and 25 mg/L chloramphenicol (25 mL in each flask) were inoculated from fresh plates of the cells co-transformed with a chaperone plasmid and SEQ ID NO:893/pET30. At inoculation the inducers required for the chaperone plasmids were also added.

Plasmid	Inducer concentration
pG-KJE8	2 mg/mL L-arabinose; 10 ng/mL tetracycline
pGro7	2 mg/mL L-arabinose
pKJE7	2 mg/mL L-arabinose
pG-Tf2	10 ng/mL tetracycline
pTf16	2 mg/mL L-arabinose

The cells were incubated at 30°C overnight and harvested by centrifugation when the OD at 600 nm reached 6 or greater. The cells were washed with cold 50 mM EPPS buffer (pH 8.4), centrifuged again, and either used immediately or frozen at –80°C.

Cell extracts were prepared by adding 5 mL per g of cell pellet of BugBuster® (primary amine-free) Extraction Reagent (EMD Biosciences/Novagen catalog #70923) with 5 μL/mL of Protease Inhibitor Cocktail II (EMD Bioscience/Calbiochem catalog #539132), 1 μl/mL of Benzonase® Nuclease (EMD Biosciences/Novagen catalog #70746), and 0.033 μl/mL of r-Lysozyme solution (EMD Biosciences/Novagen catalog #71110) to the cells. The cell suspensions were incubated at room temperature with gentle mixing for 15 min; spun at 14,000 rpm for 20 min (4°C) and the supernatants carefully removed. Total protein concentrations were determined using the Pierce BCA protein assay kit (Pierce catalog #23225) with bovine serum albumin as the standard and a microtiter plate format. The expression of the D-aminotransferase was analyzed by SDS-PAGE using Bio-Rad Ready Gel® Precast 4-15% polyacrylamide gradient gels (Bio-Rad Laboratories catalog #161-1104). BioRad SDS-PAGE low range standards (catalog #161-0304) were run as standards on each gel. Aliquots of the cell extracts (15 μg protein) were mixed with protein loading buffer containing 2% SDS, 10% glycerol, 12.5% 2-mercaptoethanol, 0.1% bromophenol blue and 62.5 mM Tris-HCl (pH 8), incubated at 95°C for 5 min, cooled and then loaded on the gel. In addition, the combined soluble and

insoluble protein expression (total protein) was analyzed for each transformant. A 10 μl aliquot of each cell suspension before centrifugation was diluted in 90 μL protein loading buffer, incubated at 95°C for 10 min, and cooled. Ten μL of each cooled solution was loaded on the gel.

The soluble protein gel showed that the best soluble expression of the polypeptide having the sequence of SEQ ID NO:894 occurred when chaperones GroEL-GroES (pGro7) were co-expressed.

The expression of the SEQ ID NO:894 polypeptide using an alternative plasmid in the presence of the GroEL-GroES chaperones was also examined. The SEQ ID NO:893 nucleic acid was subcloned into the pMET1a plasmid using the restriction enzymes *Nde*I and *Xho*I from New England Biolabs. This plasmid is a derivative of pET23a (EMD Biosciences/Novagen catalog #69745-3) and carries the *metE* gene (inserted at the *NgoM*IV site of the plasmid) and can complement the methionine auxotrophy of *E. coli* strains B834(DE3) and *E. coli* BW30384(DE3) ompTmetE ("EE2D"). The construction of the "EE2D" strain is described in WO 2006/066072. The construction of an analogous plasmid to pMET1a that is a derivative of pET23d is described in the same PCT application.

The SEQ ID NO:893/pMET1a plasmid (25 ng) was transformed into "EE2D" electrocompetent cells singly or was co-transformed with pGro7 (20 ng) using the standard Bio-Rad electroporation protocol for *E. coli* cells with a Bio-Rad Gene Pulser II system (catalog #165-2111). The transformed cells were recovered in 0.5 mL of SOC medium for 1 h at 37°C and plated on LB plates containing 100 mg/L ampicillin or on plates containing 100 mg/L ampicillin and 25 mg/L chloramphenicol (double plasmid transformants). The plates were incubated overnight at 37°C. One colony from each plate set was used to inoculate 50 mL of Novagen Overnight Express™ AutoinductionSystem 2 containing solutions 1-6, 100 mg/L ampicillin and 2 mg/mL L-arabinose. The culture inoculated with cells containing the pGro7 plasmid also contained 25 mg/L chloramphenicol. The cells were incubated at 30°C overnight and harvested by centrifugation when the OD_{600} reached 6 or greater. The cell pellets were washed with cold 50 mM EPPS buffer (pH 8.4), centrifuged again, and either used immediately or frozen at –80°C. Cell extracts were prepared as described above using the Novagen BugBuster® (primary amine-free) Extraction Reagent. The expression of soluble and total D-aminotransferase was analyzed by SDS-PAGE as described above.

The gel showed that expression of soluble SEQ ID NO:894 polypeptide was greater when the GroEL-GroES proteins were co-expressed. However, the soluble expression was not as high as with the pET30a construct described above.

The effect of incubation temperature during expression was also examined. A 5 mL culture of LB containing 100 mg/L ampicillin and 25 mg/L chloramphenicol was inoculated

from a fresh plate of EE2D::SEQ ID NO:894PMET1a+ pGro7. The culture was incubated overnight at 30°C and then used to inoculate 3 flasks, each containing 50 mL of Novagen Overnight Express™ Autoinduction System 2 containing solutions 1-6, 100 mg/mL ampicillin, 25 mg/L chloramphenicol, and 2 mg/mL L-arabinose. One flask was incubated at 20°C, the second at 25°C and the third at 30°C. The cells were harvested when the OD_{600} reached 6 or greater. The cells were harvested and cell extracts were prepared as described above. Total protein concentrations were determined using the Pierce BCA protein assay kit (Pierce catalog #23225) with bovine serum albumin as the standard and a microtiter plate format. The expression of the D-aminotransferase was analyzed using the Bio-Rad Experion Pro260 Automated Electrophoresis Station following the manufacturer's protocol with the cell extract solutions diluted to 1 mg/mL. The results are shown in Table 32. It appears that the lowest temperature gave the maximum amount of expression of the SEQ ID NO:894 polypeptide.

Table 32.

Lane	Sample	Temp	Estimated DAT Expression
1	Pro260 Ladder		
2	EE2D::23463pMET1+pGRO7 cell extract	20°C	23%
3	EE2D::23463pMET1+pGRO7 cell extract	25°C	21%
4	EE2D::23463pMET1+pGRO7 cell extract	30°C	19%

Activity Assay protocol

The enzymatic activity of the SEQ ID NO:894 DAT co-expressed with the GroEL-GroES chaperones was tested following the standard monatin reaction protocol. Briefly, each assay tube contained the following (in a total of 2 mL): 0.050 mg/mL aldolase in cell extract (assuming 20% soluble expression); 1.0 mg/mL D-aminotransferase in cell extract (assuming 20% soluble expression for an extract containing the SEQ ID NO:894 polypeptide) or purified *B. sphaericus* D-aminotransferase; 0.01 % Tween-80; 200 mM sodium pyruvate; 100 mM D-tryptophan; 100 mM EPPS (pH 8.2); 1 mM $MgCl_2$; 0.05 mM PLP; and 10 mM potassium phosphate.

The reactions were incubated at room temperature in a Coy Laboratory Products, Inc. anaerobic chamber to minimize exposure to oxygen. All components except the enzymes were mixed together (the tryptophan did not completely dissolve until at least 1 h after the addition of the enzymes). The reactions were initiated by the addition of the enzymes. Samples were withdrawn at 1, 4, 8 and 22 h. A control reaction using 1 mg/mL purified *B. sphaericus* DAT was also run. The construction, expression and purification of this DAT are described in

Example 6. The concentrations of the substrates and products were measured as described in Example 3.

The results are shown in Table 33. At 22 h, the concentration of monatin was 9.2 mM when the SEQ ID NO:894 polypeptide was present and 12.4 mM when the *B. sphaericus* enzyme was used. The concentration of the co-product HMG was significantly less when the SEQ ID NO:894 polypeptide was in the assay mixture (<1/3 the concentration when compared to the assay sample containing *B. sphaericus* enzyme). The HMG concentrations were evaluated by comparing the peak areas of the OPA derivatized samples.

Table 33. Monatin Formation (mM)

Polypeptide (SEQ ID NO)	1 h	4 h	8 h	22 h
894 DAT + GroEL-ES	1.3	4.5	6.8	9.2
B. sphaericus DAT	1.6	5.7	8.3	12.4

Example 17—Use of ArcticExpress™ system to increase the soluble expression of a DAT polypeptide

Because the soluble expression of the SEQ ID NO:894 polypeptide was low using the standard expression protocols (either 1 mM IPTG in LB or Novagen Overnight Express™ AutoinductionSystem 2—see Example 8), expression of the SEQ ID NO:893/pMET1a plasmid in the Stratagene ArcticExpress™ system was examined.

The Stratagene ArcticExpress™ system contains *E. coli* competent cells that carry the psychrophilic chaperones Cpn10 and Cpn60. These are chaperones isolated from the psychrophilic organism *Oleispira antarctica*. Cpn10 and Cpn60 show high sequence similarity to the *E. coli* chaperones GroEL and GroES, respectively, and have high protein folding activities at 4-12 °C. The ArcticExpress™ host cells are derived from the *E. coli* BL21strain. Not only do these cells lack the Lon protease, but they have been engineered to be deficient in OmpT protease as well.

Transformation protocol

The plasmid SEQ ID NO:893/pMET1a (described in Example 16) was transformed into chemically competent ArcticExpress™(DE3) cells (catalog #230192) following the manufacturer's protocol. The transformed cells were recovered in 0.5 mL of SOC medium for 1 h at 37°C and plated on LB plates containing 100 mg/L ampicillin. The plates were incubated overnight at 37°C and then stored at 4°C.

Expression protocol

Colonies from the transformation plates were used to inoculate 5 mL of 2xYT medium containing 100 mg/L ampicillin and 10 mg/L gentamycin and incubated overnight at 30°C. Flasks of Novagen Overnight Express™ AutoinductionSystem 2 (EMD Biosciences/Novagen catalog #71366) containing solutions 1-6, with 100 mg/L ampicillin and 12 mg/L gentamycin were inoculated using the overnight cultures. After incubation at 30°C for 6 h and the Overnight Express™ cultures were moved to either 15°C or 20°C or 25°C incubators. The incubations were continued until the OD at 600 nm of the cultures reached 6 or greater. The cells were harvested by centrifugation, washed with cold 50 mM EPPS, pH 8.4, and the cell pellets were frozen at –80°C.

Cell extracts were prepared by adding 5 mL per g of cell pellet of BugBuster® (primary amine-free) Extraction Reagent (EMD Biosciences/Novagen catalog #70923) with 5 μL/mL of Protease Inhibitor Cocktail II (EMD Bioscience/Calbiochem catalog #539132), 1 μl/mL of Benzonase Nuclease (EMD Biosciences/Novagen catalog #70746), and 0.033 μl/mL of r-Lysozyme™ solution (EMD Biosciences/Novagen catalog #71110) to the cells. The cell suspensions were incubated at room temperature with gentle mixing for 15 min; spun at 14,000 rpm for 20 min (4°C) and the supernatants were carefully removed. Total protein concentrations were determined using the Pierce BCA protein assay kit (Pierce catalog #23225) with bovine serum albumin as the standard and a microtiter plate format. The expression of the D-aminotransferase was analyzed using the Bio-Rad Experion Pro260 Automated Electrophoresis Station following the manufacturer's protocol with the cell extracts solutions diluted to 1 mg/mL.

The electrophoresis results show that the ArcticExpress™ system significantly increased the soluble expression of the SEQ ID NO:894 polypeptide when compared to the expression without chaperones or when co-expressed with the *E. coli* GroEL-GroES chaperones described in Example 16. The soluble expression was higher at lower temperatures, but still very high at 25°C.

Lane	Sample	Incubation Temperature	Estimated DAT Expression
1	Pro260 Ladder		
2	ArcticExpress(DE3)::894pMET1 cell extract	15°C	58%
3	ArcticExpress(DE3)::894pMET1 cell extract	20°C	46%
4	ArcticExpress(DE3)::894pMET1 cell extract	25°C	47%

Activity Assay protocol:

The enzymatic activity of the SEQ ID NO:894 polypeptide expressed in the ArcticExpress™ system was tested by following monatin formation in the presence of the aldolase described in Example 6. Each assay tube contained the following (in a total of 2 mL): 0.010 mg/mL aldolase in cell extract (assuming 20% soluble expression); 1.0 or 2.0 mg/mL of the SEQ ID NO:894 polypeptide in cell extract (assuming 50% soluble expression for the extract containing the SEQ ID NO:894 polypeptide) or purified B. *sphaericus* D-aminotransferase; 0.01 % Tween-80; 200 mM sodium pyruvate; 100 mM D-tryptophan; 100 mM EPPS, pH 8.2; 1 mM $MgCl_2$; 0.05 mM PLP; and 10 mM potassium phosphate.

The reactions were incubated at room temperature in a Coy Laboratory Products, Inc. anaerobic chamber to minimize exposure to oxygen. All components except the enzymes were mixed together (the tryptophan did not completely dissolve until at least 1 h after the addition of the enzymes). The reactions were initiated by the addition of the enzymes. Samples were withdrawn at 1, 4, 7 and 22 h. Control reactions using 1 or 2 mg/mL purified *B. sphaericus* D-aminotransferase were also run. The concentrations of the substrates and products were measured as described in Example 3. The results are shown in Table 34. At 22 h, the concentration of monatin was 8.2 mM when the SEQ ID NO:894 polypeptide was present at 1 mg/mL and 10.5 mM at 2 mg/mL DAT polypeptide. When the *B. sphaericus* enzyme was added at 1 mg/mL, the concentration of monatin at 22 h was 10.7 mg/mL; at 2 mg/mL, the monatin concentration was 14.5 mM. The stereopurity (as determined by the FDAA derivatization protocol in Example 3) of the product was > 98% R,R with both enzymes and enzyme concentrations. The concentration of the co-product HMG was significantly less when the SEQ ID NO:894 polypeptide was used (~1/3 the concentration when compared to the assay samples containing *B. sphaericus* enzyme at either enzyme concentration). The HMG concentrations were evaluated by comparing the peak areas of the OPA derivatized samples.

Table 34. Monatin Formation (mM)

Polypeptide (SEQ ID NO)	1 h	4 h	7 h	22 h
894 DAT (1 mg/mL)	0.9	2.3	3.6	8.3
894 DAT (2 mg/mL)	1.4	3.7	6.3	10.5
B. sphaericus DAT (1 mg/lmL)	1.0	4.2	6.1	10.7
B. sphaericus DAT (2 mg/lmL)	1.5	6.7	8.2	14.5

Example 18—Use of Stratagene ArcticExpress™ system to increase the soluble expression of DATs

Transformation protocol:

The plasmids SEQ ID NO:891/pET30a (encoding the SEQ ID NO:892 polypeptide), SEQ ID NO:873/pET30a (encoding the SEQ ID NO:874 polypeptide) and the *Clostridium beijerinckii* DAT (*Cb*DAT) in pET30a were transformed into chemically competent Stratagene ArcticExpress™(DE3) cells (catalog #230192) following the manufacturer's protocol. The cloning of these genes is described in Example 4 and assay results are in Example 9. The transformed cells were recovered in 0.5 mL of SOC medium for 1 h at 37°C and plated on LB plates containing 100 mg/L ampicillin and 13 mg/L gentamycin. The plates were incubated at room temperature for 2 days and then stored at 4°C.

Expression protocol:

Colonies from the transformation recovery plates were used to inoculate 5 mL of 2xYT medium containing 50 mg/L kanamycin and 13 mg/L gentamycin; the liquid cultures were incubated for 6 h at 30°C. Flasks of Novagen Overnight Express™ AutoinductionSystem 2 (EMD Biosciences/Novagen catalog #71366) containing solutions 1-6, with 100 mg/L ampicillin and 13 mg/L gentamycin were inoculated from the 5 mL cultures. After incubation at 30°C for 5-6 h, the cultures were moved to a 15°C incubator. The 15°C incubations were continued until the OD_{600} of the cultures reached 6 or greater. The cells were harvested by centrifugation, washed with cold 50 mM EPPS, pH 8.4, and then the cell pellets were frozen at –80°C.

Cell extracts were prepared by adding 5 mL per g of cell pellet of BugBuster (primary amine-free) Extraction Reagent (EMD Biosciences/Novagen catalog #70923) with 5 μL/mL of Protease Inhibitor Cocktail II (EMD Bioscience/Calbiochem catalog #539132), 1 μl/mL of Benzonase Nuclease (EMD Biosciences/Novagen catalog #70746), and 0.033 μl/mL of r-Lysozyme™ solution (EMD Biosciences/Novagen catalog #71110) to the cells. The cell suspensions were incubated at room temperature with gentle mixing for 15 min; spun at 14,000 rpm for 20 min (4°C) and the supernatants were carefully removed. Total protein concentrations were determined using the Pierce BCA protein assay kit (Pierce catalog #23225) with bovine serum albumin as the standard and a microtiter plate format. The expression of the DAT was analyzed using the Bio-Rad Experion Pro260 Automated Electrophoresis Station following the manufacturer's protocol with the cell extracts solutions diluted to 1 mg/mL. The results are shown in Tables 35 and 36.

The electrophoresis results show that the SEQ ID NO:874 polypeptide expressed in a soluble form slightly better than the SEQ ID NO:892 polypeptide using the ArcticExpress™ system. The soluble expression of the *Cb* DAT varied depending on the colony picked in the

transformation plate. None of these DAT polypeptides expressed in a soluble form using the ArcticExpress™ system as well as the SEQ ID NO:894 polypeptide described in Example 16 was expressed.

Table 35

Lane	Sample	Incubation Temperature	Estimated DAT Expression
1	Pro260 Ladder		
2	ArcticExpress(DE3)::891/pET30a cell extract (colony #1)	15°C	11%
3	ArcticExpress(DE3)::891/pET30a cell extract (colony #2)	15°C	9%
4	ArcticExpress(DE3)::873/pET30a cell extract (colony #1)	15°C	15%
5	ArcticExpress(DE3)::873/pET30a cell extract (colony #2)	15°C	10%
6	ArcticExpress(DE3)::891/pET30a cell extract (colony #1)	15°C	9%
7	ArcticExpress(DE3)::891/pET30a cell extract (colony #2)	15°C	8%
9	ArcticExpress(DE3)::873/pET30a cell extract (colony #1)	15°C	15%
10	ArcticExpress(DE3)::873/pET30a cell extract (colony #2)	15°C	11%

Table 36

Lane	Sample	Incubation Temperature	Estimated DAT Expression
1	Pro260 Ladder		
2	ArcticExpress(DE3)::*Cb*DAT in pET30a cell extract (colony #1)	15°C	9%
3	ArcticExpress(DE3)::*Cb*DAT in pET30a cell extract (colony #2)	15°C	19%
4	ArcticExpress(DE3)::*Cb*DAT in pET30a cell extract (colony #3)	15°C	13%
5	ArcticExpress(DE3)::*Cb*DAT in pET30a cell extract (colony #4)	15°C	4%

Activity assay protocol

The enzymatic activity of the DAT polypeptides expressed in the ArcticExpress™ system were tested by following monatin formation in the presence of the aldolase described in Example 6. Each assay tube contained the following (in a total of 3 mL): 0.050 mg/mL aldolase in cell extract (estimating 20% soluble expression); 0.5 mg/mL DAT polypeptide in cell extract (estimating the soluble expression from the Experion data) or purified *B. sphaericus* DAT; 0.01

% Tween-80; 200 mM sodium pyruvate; 100 mM D-tryptophan; 50 mM EPPS, pH 8.2; 1 mM $MgCl_2$; 0.05 mM PLP; and 10 mM potassium phosphate.

The reactions were incubated at room temperature in a Coy Laboratory Products, Inc. anaerobic chamber to minimize exposure to oxygen All components except the enzymes were mixed together (the tryptophan did not completely dissolve until at least 1 h after the addition of the enzymes). The reactions were initiated by the addition of the enzymes. Samples were withdrawn at 2, 4.5, 9 and 24 h. Control reactions with purified *B. sphaericus* D-aminotransferase were also run. The concentrations of the substrates and products were measured as described in Example 3. At 24 h, the assays containing the SEQ ID NO:892 or 894 polypeptide had produced approximately the same titer of monatin as the control *B. sphaericus* DAT reaction. The *C. beijerinckii* DAT reaction produced less than one-eighth of the monatin product, while the SEQ ID NO:874 polypeptide produced about half. The stereopurity of the product at 24 h (as determined by the FDAA derivatization protocol in Example 3) was 96% R,R monatin or greater for the SEQ ID NO:892 polypeptide. The concentration of the by-product HMG (4-hydroxy-4-methyl glutamic acid) was measured for the reactions with the SEQ ID NO:892, the SEQ ID NO:894 and the *B. sphaericus* DAT polypeptides. The assay with polypeptides having the sequence shown in SEQ ID NO:894 produced far less of the HMG by-product than the other two (about 20% of that produced by the assay containing the *B. sphaericus* DAT and about 40% of that produced by the assay with the SEQ ID NO:892 polypeptide). The HMG concentrations were estimated by comparing the peak areas of the OPA post-column derivatized samples.

Table 37. Monatin Formation (mM)

Polypeptide (SEQ ID NO)	2 h	4.5 h	9 h	24 h
C. beijerinckii DAT	0.3	0.7	0.8	0.9
874 DAT	1.8	3.2	4.1	4.4
892 DAT	1.6	3.7	5.4	8.4
894 DAT	1.4	2.8	4.5	8.4
B. sphaericus DAT	1.2	2.9	4.3	8.1

These results indicate that when expressed under the appropriate conditions, the polypeptides having the sequence of SEQ ID NO:892 and 894 can be utilized in reactions to produce highly pure R,R monatin at a titer as high as the positive control enzyme.

Example 19—Evaluation of alternative expression hosts to increase the soluble expression of a DAT

Because the soluble expression of the SEQ ID NO:894 polypeptide was low when the nucleic acid was expressed in BL21(DE3) (see Example 8), alternative expression hosts were evaluated for improving soluble expression. The OverExpress™ C41(DE3) and C43(DE3) hosts contain genetic mutations phenotypically selected for conferring toxicity tolerance and express some toxic proteins at higher titers than other *E. coli* hosts.

Transformation protocol

The plasmid SEQ ID NO:893/pET30a was transformed into electrocompetent cells of the OverExpress™ C41(DE3) and C43(DE3) (Lucigen catalog #60341 and 60345, respectively) using a Bio-Rad Gene Pulsar II system following the manufacturer's protocol. The transformation mixtures were recovered in 1 mL SOC medium for 1 hr at 37°C and plated on LB plates containing 50 mg/L kanamycin. The plates were incubated overnight at 37°C. Multiple colonies were patched onto fresh plates and analyzed for the appropriate insert size using colony PCR. A small aliquot of cells was suspended in 0.025 mL H_2O and incubated at 95°C for 10 min. After cooling, 2 μL of each of the suspensions were used as the template in 0.025 mL reactions, each also containing 0.5 μL T7 promoter primer (0.1 mM), 0.5 μL T7 terminator primer (0.1 mM), 0.5 μL PCR Nucleotide Mix (Roche #12526720; 10 mM each nucleotide), 2.5 μL Roche Expand DNA polymerase buffer #2, and 0.5 μL Expand DNA polymerase (Roche Expand High Fidelity PCR System catalog #1732650). The 3-step thermocycler program was run for 25-30 cycles: 1 min at 94 °C; 1 min at 54 °C, 1.3 min at 72 °C with a final polishing step of 7 min at 72 °C.

Expression studies

Flasks of Novagen Overnight Express™ AutoinductionSystem 2 (EMD Biosciences/Novagen catalog #71366) containing solutions 1-6 and 50 mg/L kanamycin (40 mL in each flask) were inoculated from the patch plates of the transformed C41(DE3) and C43(DE3) cells (2 patches for each of the transformations). The cells were incubated at 30°C overnight and harvested by centrifugation when the OD_{600} reached 6 or greater. The cells were washed with cold buffer, were centrifuged again, and either used immediately or frozen at –80°C.

Cell extracts were prepared by adding 5 mL per g of cell pellet of BugBuster® (primary amine-free) Extraction Reagent (EMD Biosciences/Novagen catalog #70923) with 5 μL/mL of Protease Inhibitor Cocktail II (EMD Bioscience/Calbiochem catalog #539132), 1 μl/mL of Benzonase® Nuclease (EMD Biosciences/Novagen catalog #70746), and 0.033 μl/mL of r-Lysozyme™ solution (EMD Biosciences/Novagen catalog #71110) to the cells. The cell

suspensions were incubated at room temperature with gentle mixing for 15 min; spun at 14,000 rpm for 20 min (4°C) and the supernatants carefully removed. Total protein concentrations were determined using the Pierce BCA protein assay kit (Pierce catalog #23225) with bovine serum albumin as the standard and a microtiter plate format. The expression of the DAT polypeptide was analyzed by SDS-PAGE using Bio-Rad Ready Gel^R Precast 4-15% polyacrylamide gradient gels (Bio-Rad Laboratories catalog #161-1104). BioRad SDS-PAGE low range standards (catalog #161-0304) were run as standards on each gel. Aliquots of the cell extracts (15 μg protein) were mixed with protein loading buffer containing 2% SDS, 10% glycerol, 12.5% 2-mercaptoethanol, 0.1% bromophenol blue and 62.5 mM Tris-HCl (pH 8), incubated at 95°C for 5 min, cooled and then loaded on the gel. In addition, the combined soluble and insoluble protein expression (total protein) was analyzed for the transformants. A 10 μl aliquot of each cell suspension before centrifugation was diluted in 90 μL protein loading buffer, incubated at 95°C for 10 min, and cooled. Ten μL of each cooled solution was loaded on the gel.

The electrophoresis gel shows that the protein expressed better in the C41(DE3) host than in the C43(DE3) host, however the apparent soluble expression was not higher than when BL21(DE3) cells were used.

Example 20—Evaluation of low temperature expression to increase the soluble expression of DAT

Because the soluble expression of SEQ ID NO:894 D-aminotransferase was low when the gene was expressed in the *E. coli* strain BL21(DE3) (see Example 8), the gene was inserted in vectors with cold shock Protein A promoters to evaluate low temperature expression.

The Takara pCold Expression Vectors are four different vectors that utilize the cold shock Protein A (*cspA*) promoter for expression of high purity, high yield recombinant protein in *E. coli*. These vectors selectively induce target protein synthesis at low temperatures (15°C) where the synthesis of other proteins is suppressed and protease activity is decreased. In addition to the *cspA* promoter, all four vectors contain a *lac* operator (for control of expression), ampicillin resistance gene (*amp*^{r}), ColE1 origin of replication, M13 IG fragment, and multiple cloning site (MCS). Three of the vectors also contain a translation enhancing element (TEE), a His-Tag sequence, and/or Factor Xa cleavage site.

Cloning protocol

The SEQ ID NO:893 DAT nucleic acid from plasmid SEQ ID NO:893/pET30a (encoding the polypeptide having the sequence of SEQ ID NO:894) was subcloned into the Takara pCold vectors at the *Nde*I and *Xho*I sites of the pCOLDII (contains a TEE and a His-tag sequence), pCOLDIII (contains a TEE) and pCOLDIV vectors. The digested vector and insert

bands were gel purified using a QIAquick Gel Extraction Kit (Qiagen catalog #28704) and ligated using a Roche Rapid DNA Ligation Kit (catalog # 1635379) following the manufacturer's protocol. The ligation mixtures were transformed into Invitrogen OneShot TOP10 chemically competent cells (catalog # C404003) by heat shock at 42°C. After recovery in 500 μL SOC medium for 1 h at 37°C, the transformation mixtures were plated on LB plates containing 100 mg/L ampicillin and incubated at 37°C overnight. Colonies were picked from the transformation plates and used to inoculate 5 mL cultures of LB containing 100 mg/mL ampicillin that were incubated overnight at 37°C. Plasmid DNA was purified from the 5 mL cultures using a QIAprep^{R} Spin Miniprep Kit (Qiagen catalog #27104). The inserts were verified restriction digestion with *Nde*I and *Xho*I by sequencing (Agencourt Bioscience Corp, Beverly, MA).

The SEQ ID NO:894*dat* pCOLD plasmids were transformed into chemically competent Stratagene ArcticExpress^{TM}(DE3) cells and Novagen BL21(DE3) cells following the manufacturers' protocols. The transformation mixtures were recovered in 0.5-1 mL SOC medium for 1 h at 37°C and plated on LB plates containing 100 mg/mL ampicillin and 13 mg/L gentamycin (ArcticExpress^{TM}(DE3)) or 100 mg/mL ampicillin (BL21(DE3)). The plates were incubated overnight at 37°C.

Expression studies

Flasks of Novagen Overnight Express^{TM} AutoinductionSystem 2 (EMD Biosciences/Novagen catalog #71366) containing solutions 1-6 and 100 mg/L ampicillin and 13 mg/L gentamycin (ArcticExpress^{TM}(DE3)) or 100 mg/mL ampicillin (BL21(DE3) were inoculated from the patch plates of the transformed cells (2 patches for each of the SEQ ID NO:893/pCOLDII, SEQ ID NO:893/pCOLDIII and SEQ ID NO:893/pCOLDIV transformations).

After incubation at 30°C for 3-5 hr the cultures were moved to a 15°C incubator. The 15°C incubations were continued until the OD at 600 nm of the cultures reached 6 or greater. The cells were harvested by centrifugation, washed with cold buffer, and then the cell pellets were frozen at –80°C.

Cell extracts were prepared by adding 5 mL per g of cell pellet of BugBuster^{R} (primary amine-free) Extraction Reagent (EMD Biosciences/Novagen catalog #70923) with 5 μL/mL of Protease Inhibitor Cocktail II (EMD Bioscience/Calbiochem catalog #539132), 1 μl/mL of Benzonase^{R} Nuclease (EMD Biosciences/Novagen catalog #70746), and 0.033 μl/mL of r-Lysozyme^{TM} solution (EMD Biosciences/Novagen catalog #71110) to the cells. The cell suspensions were incubated at room temperature with gentle mixing for 15 min; spun at 14,000 rpm for 20 min (4°C) and the supernatants were carefully removed. Total protein concentrations

were determined using the Pierce BCA protein assay kit (Pierce catalog #23225) with bovine serum albumin as the standard and a microtiter plate format.. The expression of the D-aminotransferase was analyzed using the Bio-Rad Experion™ Pro260 Automated Electrophoresis Station following the manufacturer's protocol with the cell extract solutions diluted to 1 mg/mL. The results are shown in Tables 38 and 39.

Table 38

Lane	Sample	Estimated % DAT Expression
L	Pro260 Ladder	
1	BL21(DE3)::SEQ ID NO:893/pCOLDII#1	8.7
2	BL21(DE3):: SEQ ID NO:893/pCOLDII#2	8.5
3	ArcticExpress™(DE3):: SEQ ID NO:893/pCOLDII#1	9.8
4	ArcticExpress™(DE3):: SEQ ID NO:893/pCOLDII#2	6.4

Table 39

Lane	Sample	Estimated % DAT Expression
L	Pro260 Ladder	
1	BL21(DE3):: SEQ ID NO:893/pCOLDIII#1	4.3
2	BL21(DE3):: SEQ ID NO:893/pCOLDIII#2	2.3
3	BL21(DE3):: SEQ ID NO:893/pCOLDIV#1	14.6
4	BL21(DE3):: SEQ ID NO:893/pCOLDIV#2	14.2
5	BL21(DE3):: SEQ ID NO:893/pCOLDIII#1	4.4
6	BL21(DE3):: SEQ ID NO:893/pCOLDIII#2	5.2
7	BL21(DE3):: SEQ ID NO:893/pCOLDIV#1	13.8
8	BL21(DE3):: SEQ ID NO:893/pCOLDIV#2	16.1
9	BL21(DE3):: SEQ ID NO:893/pCOLDIV#1	16.2
10	BL21(DE3):: SEQ ID NO:893/pCOLDIV#2	16.8

The Experion Pro260 results show that the SEQ ID NO:894 DAT protein expressed better when the nucleic acid was incorporated in the pCOLDIV vector than in either the pCOLDII or pCOLDIII vector. From the experiments shown above, the average expression level for SEQ ID NO:893/pCOLDII was about 8%, regardless of expression host used; the average expression level for SEQ ID NO:893/pCOLDIII was about 4%, while the average expression level for SEQ ID NO:893/pCOLDIV was ~15%. These expression levels are significantly less than those described in Examples 16 when the SEQ ID NO:893 nucleic acid was co-expressed with the GroEL-GroES chaperones and in Example 17 when the nucleic acid was expressed in the Stratagene ArcticExpress™ system.

Example 21—Codon Modification of a DAT

An attempt to improve the solubility of the SEQ ID NO:894 polypeptide expressed in *E.coli* was undertaken with the presumption that, slowing the rate of translation in *E.coli* would allow more time for proper folding of the SEQ ID NO:894 polypeptide, thereby giving a higher expression of soluble protein. A BLAST search (NCBI) of the SEQ ID NO:894 polypeptide sequence revealed that some of the most closely related public sequences were from *Clostridium* species, specifically *Clostridium beijerinckii.* Example 9 describes the results from cloning, expressing, and assaying the *Cb*DAT and its use in monatin formation reactions. Specifically, expression was high in the total protein fraction but very low in the soluble protein fraction.

The codon usage tables of *C. beijerinckii* and *E. coli* K12 were compared. Several rarely used codons in *C. beijerinckii* were found to be highly abundant in *E.coli* K12 (Table 40). It is possible that these rare codons cause translational pauses in *C. beijerinckii.*, whereas in an *E.coli* K12 host, there may not be a pause. In the SEQ ID NO:894 sequence, 4 doublets were identified in which tandem rare codons for *C. beijerinckii* had become "non-rare" (i.e. abundant) in *E. coli* K12. The goal was to change these codons into rare codons for expression in *E.coli* K12 host using the *E.coli* K12 codon usage table. Primers were designed to change these doublets. SEQ ID NO:893/pET30a (described in Example 4) was used as a template and mutation was carried out according to the Stratagene QuickChange kit instructions. The primers utilized to modify the SEQ ID NO:893 nucleic acid sequence are shown below, along with the native gene (the targeted doublet sequences are underlined).

Table 40.

Original Codons	Codon Usage (per thousand)		Altered Codons	Codon Usage
	C. beijerinckii	*E. coli*		*E. coli*
GCC	3.7	25.6	GCT	15.3
CTG	1.4	52.9	CTA	3.9
ACC	2.5	23.5	ACA	7.0
CGC	0.8	22.0	CGA	3.5
GCG	2.9	33.8	GCT	15.3
CCG	1.1	23.3	CCC	5.4

```
>SEQ ID NO:893 native sequence
        ATGGACGCACTGGGATATTACAACGGAAAATGGGGGCCTCTGGACGAGATGACCGTGCCGATGAACGACAG
GGGTTGTTTCTTTGGGGACGGAGTGTACGACGCTACCATCGCCGCTAACGGAGTGATCTTTGCCCTGGACGAGCACA
TTGACCGGTTTTTAAACAGCGCAAAGCTCCTGGAAATAGAAATCGGTTTTACAAAAGAGGAATTAAAAAAAACTTTT
TTTGAAATGCACTCCAAAGTGGATAAAGGGGTGTACATGGTTTATTGGCAGGCGACTCGCGGAACAGGCCGTCGAAG
CCATGTATTTCCGGCAGGTCCCTCAAATCTCTGGATTATGATTAAGCCCAATCACGTCGACGATCTTTATAGAAAAA
TCAAGCTCATTACCATGGAAGATACCCGCTTCCTCCACTGCAACATCAAGACCCTTAACCTTATTCCCAATGTCATT
GCCTCCCAGCGGGCGCTGGAAGCGGGCTGCCACGAGGCGGTCTTTCACCGGGGTGAAACAGTAACCGAGTGCGCCCA
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CAGCAATGTCCACATCATTAAAAACGGCAGGTTTATCACCCACCAGGCGGACAACCTAATCCTTCGGGGCATAGCCC
GTAGCCATTTATTGCAAGCCTGTATCAGGCTGAACATTCCATTTGACGAACGGGAATTTACCCTTTCGGAATTATTT
GACGCGGATGAGATTCTTGTGTCCAGCAGCGGCACACTCGGCCTTAGCGCCAATACAATTGATGGAAAAAACGTGGG
GGGAAAAGCGCCGGAACTGCTAAAAAAAATTCAGGGCGAAGTGTTGAGGGAATTTATCGAAGCGACAGGCTACACGC
CTGAGTGGAGCACAGTATAG

Primers for Doublet 1 mutant

CTAACGGAGTGATCTTTGCTCTAGACGAGCACATTGAC (SEQ ID NO:1074)

GTCAATGTGCTCGTCTAGAGCAAAGATCACTCCGTTAG (SEQ ID NO:1075)

Primers for Doublet 2 mutant

CATGGAAGATACACGATTCCTCCACTGCAACATCAAGAC (SEQ ID NO:1076)

GTCTTGATGTTGCAGTGGAGGAATCGTGTATCTTCCATG (SEQ ID NO:1077)

Primers for Doublet 3 mutant

ATTGCCTCCCAGCGGGCTCTAGAAGCGGGCTGCCACG (SEQ ID NO:1078)

CGTGGCAGCCCGCTTCTAGAGCCCGCTGGGAGGCAAT (SEQ ID NO:1079)

Primers for Doublet 4 mutant

GGGGGGAAAAGCTCCCGAACTGCTAAAAAAAATTCAGG (SEQ ID NO:1080)

CCTGAATTTTTTTTAGCAGGTCGGGAGCTTTTCCCCCC (SEQ ID NO:1081)

Clones with the correctly altered sequence were transformed into BL21(DE3) host for enzyme expression assays. Enzyme expression was determined by growing the cells overnight in Overnight Express II and lysing the cells with BugBuster reagent followed by SDS PAGE analyses of crude cell extract and soluble protein.

It appeared that there was a slight improvement in soluble protein expression with codon changes to doublets 1, 2 and 3. Codon changes at doublet 4 were not beneficial for soluble protein expression. Codon changes for doublets 1, 2 and 3 were combined in pairs using the Stratagene QuickChange kit and the primers designed for the initial codon changes. Clones with the correctly altered sequence were transformed into BL21(DE3) host for enzyme expression assays. Enzyme expression was determined by growing the cells overnight in Overnight Express II and lysing the cells with BugBuster reagent followed by SDS PAGE analyses of crude cell extract and soluble protein. The combinations of mutations to doublets 1 and 2, 2 and 3 and 1 and 3 yielded soluble protein bands visible on an SDS-PAGE gel. However, there still appeared to be more protein in the total protein fractions.

Example 22—The evaluation of periplasmic expression of a DAT polypeptide

Because the soluble expression of the SEQ ID NO:894 polypeptide was low when the gene product was expressed as a cytoplasmic protein in the *E. coli* host BL21(DE3) (see Example 8), the gene was cloned into vectors to generate fusion proteins that should be exported into the periplasmic space. The periplasm provides conditions that promote proper folding and disulfide bond formation and may enhance the solubility and activity of certain target proteins.

Cloning into EMD Biosciences/Novagen pET26b allows production of the target protein with a periplasmic signal sequence. The signal sequence is cleaved by signal peptidase concomitant with export. The EMD Biosciences/Novagen pET39b and pET40b are designed for cloning and expression of target proteins fused with a 208 amino acid DsbA-Tag™ or 236 amino acid DsbC-Tag™. DsbA and DsbC are periplasmic enzymes that catalyze the formation and isomerization of disulfide bonds, respectively. The fusion proteins are typically localized in the periplasm.

Cloning protocol

The SEQ ID NO:893 nucleic acid from plasmid SEQ ID NO:893/pET30a (described in Example 4) was cloned into the EMD Biosciences/Novagen pET26b (catalog #69862-3), pET39b (catalog #70090-3), and pET40b (catalog #70091-3) vectors at the *EcoR*I and *Not*I sites of the vectors. The DATs nucleic acid with a 5' *EcoR*I site and a 3' *Not*I site was generated using the amplification protocol described in Example 4 and the following primers:

5'-CGCA***GAATTC***GGACGCACTGGGATATTACAAC-3' (SEQ ID NO:1082)

5'-GTTA***GCGGCCGCC***TATACTGTGCTCCACTCAG-3' (SEQ ID NO:1083)

The restriction sites are in italics in the primer sequences. The resulting DNA product and the pET26b, pET39b and pET40b vectors were digested with *EcoR*1 and *Not* I (New England Biolabs) following the suggested manufacturer's protocol. The vector reaction mixtures were subsequently treated with Shrimp Alkaline Phosphatase (Roche catalog # 1758250). The digested vector and insert bands were gel purified from a 1% agarose gel using a Qiagen QIAquick® Gel Extraction Kit (catalog # 28704) and ligated using a Roche Rapid Ligation Kit (catalog # 1635379) following the manufacturer's protocol. The ligation mixtures were transformed into Invitrogen OneShot® TOP10 chemically competent cells (catalog # C404003) by heat shock at 42°C. After recovery in 500 μL SOC medium for 1 h at 37°C, the transformation mixtures were plated on LB plates containing 50 mg/L kanamycin and incubated at 37°C overnight. Colonies were picked from the transformation plates and used to inoculate 5 mL cultures of LB containing 50 mg/mL kanamycin that were incubated overnight at 37°C. Plasmid DNA was purified from the 5 mL cultures using a Qiagen QIAprep spin miniprep kit (catalog # 27104). The nucleic acid sequences were verified by sequencing (Agencourt

Bioscience Corp, Beverly, MA). Plasmids with the correct insert sequences were transformed into EMD Biosciences/Novagen BL21(DE3) chemically competent cells (catalog # 69450) by heat shock as described above.

Expression studies

Flasks of Novagen Overnight Express™ AutoinductionSystem 2 (EMD Biosciences/Novagen catalog #71366) containing solutions 1-6 and 50 mg/L kanamycin (50 mL in each flask) were inoculated from fresh plates of BL21(DE3) cells carrying the SEQ ID NO:893 DAT nucleic acid (encoding the polypeptide of SEQ ID NO:894) in either pET26b, pET39b or pET40b. The cells were incubated at 30°C overnight and harvested by centrifugation when the OD_{600} reached 6 or greater. The cells were washed with cold buffer, were centrifuged again, and used immediately or the cell pellets were frozen at –80°C. Before harvesting, 2 mL culture aliquots were withdrawn from each flask for soluble and total protein (soluble and insoluble) expression analyses. Cell extracts were prepared as described in Example 16. Total protein samples were prepared by suspending a small amount of cell pellet in protein loading buffer containing 2% SDS, 10% glycerol, 12.5% 2-mercaptoethanol, 0.1% bromophenol blue and 62.5 mM Tris-HCl, pH 8, and incubating at 95°C for 10 min.

The periplasmic cellular fractions were prepared from the remainder of the cells from each culture following the protocol described in the EMD Biosciences/Novagen pET System Manual. The resulting fractions were concentrated 30-fold using Amicon Ultracel 10k centrifugal filter devices (Millipore catalog #UFC901024). Total protein concentrations of the cell extracts and the periplasmid fractions were determined using the Pierce BCA protein assay kit (Pierce catalog #23225) with Bovine Serum Albumin as the standard and a microtiter plate format. Fifteen μg protein samples of the cell extracts and 10 μg protein samples of the periplasmic fractions were analyzed by SDS-PAGE using Bio-Rad Ready Gel® Precast 4-15% polyacrylamide gradient gels (Bio-Rad Laboratories catalog #161-1104). In addition, the total protein samples were analyzed by SDS-PAGE. BioRad SDS-PAGE low range standards (catalog #161-0304) were run as standards on each gel.

Analysis of the total protein SDS-PAGE gel shows that proteins with the predicted molecular weights expressed using the Overnight Express™ AutoinductionSystem 2. However, analysis of the SDS-PAGE gel loaded with the cell extract fractions or with the periplasmic fractions suggests that these proteins did not express solubly nor were they exported into the periplasm.

Example 23—Production of monatin from indole-3-pyruvate

The maximum concentration of monatin obtained when D-tryptophan and pyruvic acid are the starting raw materials in the monatin formation assay described in Example 5 is limited by the solubility of tryptophan. In order to explore the potential of using an aldolase and the SEQ ID NO:220 DAT polypeptide (described in Example 14) in reaching higher monatin concentrations, the reaction starting with indole-3-pyruvatr (I3P) and pyruvate acid as raw materials were analyzed. In this case, it was also necessary to provide an amino donor such as D-alanine or both D-alanine and D-tryptophan.

The test was conducted using purified SEQ ID NO:220 DAT polypeptide (production and purification described in Example 15) and an aldolase (described in Example 6). The reaction was set up as follows (in a total of 1 mL): 200 mM Indole-3-pyruvate (I3P); 200 mM sodium pyruvate; 400 mM D-alanine; 100 mM EPPS, pH 8.0; 1 mM $MgCl_2$; 0.05 mM PLP; and 10 mM potassium phosphate.

Both enzymes were added in excess to facilitate conversion to monatin to minimize competion from non-enzymatic degradation reactions. The reactions were incubated at room temperature in a Coy Laboratory Products, Inc. anaerobic chamber to minimize exposure to oxygen All components except the enzymes were mixed together and the pH was adjusted to 8.0. The reactions were initiated by the addition of the enzymes (0.04 mg/mL aldolase as cell extract (assuming 20% expression) and 0.40 mg/mL purified SEQ ID NO:220 DAT polypeptide).

In some tests as indicated in the table below, D-tryptophan was also added at either 50 or 100 mM in addition to the D-alanine to act as amino donor and also to limit the amount of indole-3-pyruvate consumed in the formation of D-tryptophan. The monatin formation was measured after 18 hours using the LC/MS/MS methodology described in Example 3, and the results are presented in Table 41 below.

Table 41. Monatin Formation from I3P (mM)

Reactant initial concentrations (mM)	Monatin concentration (mM)
200 I3P; 200 pyr: 400 D-ala	44.9
200 I3P; 200 pyr: 400 D-ala, 50 D-trp	47.8
200 I3P; 200 pyr: 400 D-ala, 100 D-trp	61.0

As shown above, the aminotransferase and aldolase enzymes were active at the higher reactant concentrations and a much higher monatin concentration was achieved.

At 18 h, while using 200 mM indole-3-pyruvate, 200 mM sodium pyruvate and 100 mM D-tryptophan, the concentration of monatin was 61 mM. A small increase in monatin production

(47.8 mM) was observed under the conditions of the assay, with the addition of 50 mM D-tryptophan vs. just using 400 mM D-alanine.

Example 24—Homology Table

Table 42 shows some of the most active DAT polypeptides and the corresponding closest homologs from the published databases or literature.

Table 42

DAT polypeptide (SEQ ID NO)	Closest Hit from Database	% Sequence Identity
896	Bacillus sp. YM-1	76
874	Serine glyoxylate transaminase from *Acidiphilium cryptum* JF-5 (NR Accession No: 148260372)	51
878	Putative glutamate-1-semialdehyde 2,1-aminomutase from *Planctomyces maris* DSM 8797 (NR Accession No. 149173540)	43
882	D-alanine transaminase from *Oceanobacter* sp. RED65 (NR Accession No: 94500389)	57
910	DAT from *B. macerans*	91
176	D-amino acid aminotransferase from *Clostridium beijerincki* (NCIMB 8052)	62
220	D-amino acid aminotransferase from *Clostridium beijerincki* (NCIMB 8052)	62
156	Aminotransferase class-III (leadered) from *Chloroflexus aggregans* DSM 9485 (NR Accession No: 118045454)	46
214	D-amino acid aminotransferase from *Clostridium beijerincki* (NCIMB 8052)	61
918	Aminotransferase class IV from *Robiginitalea biformata* HTCC2501 (NR Accession No: 88806011)	57
902	Putative glutamate-1-semialdehyde 2;1-aminomutase from *Planctomyces maris* DSM 8797 (same protein as above)	46
884	D-alanine transaminase from *Thiobacillus denitrificans* ATCC 25259 (NR Accession No: 74316285)	40
866	D-alanine aminotransferase from *Lactobacillus salivarius* subsp. *salivarius* UCC118	49
946	D-amino acid aminotransferase from *Clostridium beijerincki* (NCIMB 8052)	63

As shown in Example 9, homologs of the polypeptides having the sequence shown in SEQ ID NO:866, 946, 220, and 176 were cloned and also had activity in the production of R,R monatin, despite a sequence identity among those polypeptides of between 49% and 63%. Similarly, the predicted D-alanine transaminase from *Oceanobacter* species and the *Robinginitalea biformata* aminotransferase are expected to have broad D-amino acid aminotransferase activity like the DAT polypeptides having the sequence of SEQ ID NO:882 and 918.

Appendix I shows a table that describes selected characteristics of exemplary nucleic acids and polypeptides of the invention, including sequence identity comparison of the exemplary sequences to public databases. By way of example and to further aid in understanding Appendix I, the first row, labeled "SEQ ID NO:", the numbers "1, 2" represent the exemplary polypeptide of the invention having a sequence as set forth in SEQ ID NO:2, encoded by, e.g., SEQ ID NO:1. The sequences described in Appendix I (the exemplary sequences of the invention) have been subject to a BLAST search (as described herein) against two sets of databases. The first database set is available through NCBI (National Center for Biotechnology Information). The results from searches against these databases are found in the columns entitled "NR Description", "NR Accession Code", "NR E-value" or "NR Organism". "NR" refers to the Non-Redundant nucleotide database maintained by NCBI. This database is a composite of GenBank, GenBank updates, and EMBL updates. The entries in the column "NR Description" refer to the definition line in any given NCBI record, which includes a description of the sequence, such as the source organism, gene name/protein name, or some description of the function of the sequence. The entries in the column "NR Accession Code" refer to the unique identifier given to a sequence record. The entries in the column "NR E-value" refer to the Expected value (E-value), which represents the probability that an alignment score as good as the one found between the query sequence (the sequences of the invention) and that particular database sequence would be found in the same number of comparisons between random sequences as was done in the present BLAST search. The entries in the column "NR Organism" refer to the source organism of the sequence identified as the closest BLAST hit.

The second database set is collectively known as the GENESEQ™ database, which is available through Thomson Derwent (Philadelphia, PA). The results from searches against this database are found in the columns entitled "GENESEQ Protein Description", "GENESEQ Protein Accession Code", "E-value", "GENESEQ DNA Description", "GENESEQ DNA Accession Code" or "E-value". The information found in these columns is comparable to the information found in the NR columns described above, except that it was derived from BLAST searches against the GENESEQ™ database instead of the NCBI databases.

In addition, this table includes the column "Predicted EC No.". An EC number is the number assigned to a type of enzyme according to a scheme of standardized enzyme nomenclature developed by the Enzyme Commission of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB). The results in the "Predicted EC No." column are determined by a BLAST search against the Kegg (Kyoto Encyclopedia of Genes and Genomes) database. If the top BLAST match has an E-value equal to or less than e-6, the EC number assigned to the top match is entered into the table. The columns "Query DNA Length" and "Query Protein Length" refer to the number of nucleotides or the number amino acids, respectively, in the sequence of the invention that was searched or queried against either the NCBI or GENESEQ™ databases. The columns "Subject DNA Length" and "Subject Protein Length" refer to the number of nucleotides or the number amino acids, respectively, in the sequence of the top match from the BLAST searches. The results provided in these columns are from the search that returned the lower E-value, either from the NCBI databases or the GENESEQ™ database. The columns "%ID Protein" and "%ID DNA" refer to the percent sequence identity between the sequence of the invention and the sequence of the top BLAST match. The results provided in these columns are from the search that returned the lower E-value, either from the NCBI databases or the GENESEQ™ database.

Part C

Example 25—Construction and Testing of GSSM^{SM} Mutants

This example describes the construction of exemplary nucleic acids and polypeptides, and describes their enzymatic activity. The nucleotide sequence (SEQ ID NO:219) was subcloned into pSE420-C-His vector and expressed in *E. coli* XL1-BLUE host (Stratagene, La Jolla, CA) to produce the exemplary D-aminotransferase (DAT) enzyme having the amino acid sequence shown in SEQ ID NO:220. The pSE420-C-His vector was created by adding a C-terminal His-tag to the pSE420 vector from Invitrogen (Carlsbad, CA). Construct SEQ ID NO:220 (in *E. coli* XL1-BLUE), was used as a starting sequence into which modifications were introduced and is referred to herein as the wild type (WT) sequence. A first round of modification (i.e., single-residue mutations) was performed using Gene Site Saturated Mutagenesis^{SM} (GSSM^{SM}) technology (see, for example, U.S. Patent No. 6,171,820). The mutants made using GSSM^{SM} technology were expressed in the pSE420-C-His vector in *E. coli* host XL1-BLUE, arrayed into 384-well plates and grown at 37°C overnight. Samples were subcultured and grown at 30°C for two nights (36-48 hours). Cultures were frozen at -20°C until cell lysates could be prepared.

Cells were lysed by addition of 10 μL of BPER II (Thermo Scientific, Rockford, IL) to each well. Samples were mixed three times and lysed on ice for one hour. Crude lysates were then assayed in the primary screen. 25 μL of crude lysate was assayed with 1 mM R,R-monatin, 25 mM pyruvic acid sodium salt, 0.08 mM PLP in 50 mM sodium phosphate (pH 8) at room temperature. After three hours, an aliquot was taken and formic acid was added to a final concentration of 2%. Samples were diluted with water to be within the range of the standard curve. Samples were analyzed for monatin consumption and alanine formation using the LC/MS/MS methods described in Example 1 (LC/MS/MS Method for Detecting D-alanine or R,R-monatin). Sample performance was compared to the performance of the wild type control (i.e., SEQ ID NO:220).

Mutants that outperformed the wild type control were selected as hits from the GSSM^{SM} primary screen. Glycerol stocks of the primary hits were used to inoculate new 384-well plates. The hits were arrayed in quadruplicate, grown and lysed as indicated for the primary screen. The primary hits were then tested in a secondary screen. The secondary screen method was the same as for the primary screen except the mutants were tested with 1 mM and 15 mM R,R-monatin substrate. Samples were analyzed for monatin consumption and alanine formation using LC/MS/MS. Sample performance was compared to the performance of the wild type control.

Sample performance was evaluated using a scoring system based on alanine production and monatin consumption. The maximum score for a single sample was six. A maximum of three points were assigned for alanine production and a maximum of three points were assigned for monatin consumption. The scoring criteria were as follows: 1 point assigned for a value between average and one standard deviation of the positive control; 2 points assigned for a value between one and two standard deviations of the positive control; and 3 points assigned for a value beyond two standard deviations of the positive control.

The highest potential total score for a mutant was 48 (since the samples were screened in quadruplicate at 1 and 15 mM monatin). In general, mutants scoring 20 points or more were selected as secondary hits. However, some exceptions were made for samples scoring less than 20 points. Samples with alanine formation and monatin consumption values on the verge of the threshold requirements were also selected as hits. This prevented the premature elimination of hits and allowed for further testing and characterization in the tertiary screen.

Samples identified as secondary screen hits, using the criteria above, are listed in Table 43. Secondary hits were streaked from glycerol stocks onto LB agar plates containing 100 μg/mL carbenicillin and grown overnight at 37°C. Single colonies were used to inoculate 1 mL LB containing carbenicillin (100 μg/mL). Cultures were grown overnight at 37°C. DNA was

isolated from the cultures, and then prepared and sequenced using 3730XL automated sequencers (Applied Biosystems, Foster City, CA).

Mutations and the amino acid position of the mutation for secondary hits are listed below in Table 43. Numbering of the amino acid positions starts with the N-terminal methionine. For example, the first mutation listed "Y6L" refers to changing the tyrosine in amino acid position 6 of the wild type enzyme (SEQ ID NO:220) to leucine. At the nucleic acid level, any codon which codes for the desired (mutated) amino acid can be used.

All of the amino acid sequences described in Tables 43, 44 and 50, below, are exemplary polypeptides; also provided are nucleic acid sequences that encode such polypeptides.

Example 26—List of GSSM^{SM} Mutations

Table 43: GSSM^{SM} Mutants Identified as Secondary Screen Hits

Mutant name	Mutation	Mutant name	Mutation
1	Y6L	92	D2G
2	Y6C; SILENT MUTATION AT AA31 (GGC → GGT)	93	D2Q
3	Y6F	94	D2F
4	Y6L	95	D2A
5	Y6H	96	D2T
6	Y6L	97	D2N
7	Y6M	98	D2R
8	N10S	99	D2I
9	N10W	100	D2V; G9A
10	N10T	101	G12A
11	N10R	102	D47W
12	N10T	103	S56S
13	L14V	104	I64H
14	L14L	105	L66L
15	G41G	106	I64C
16	T18W	107	L66G
17	N40N	108	E69Y
18	V19T	109	T74L
19	V42V	110	K73L
20	I62C	111	T74V
21	V82A	112	T74M
22	A57M	113	T74R
23	V42M	114	T74A
24	G41Y	115	N76C
25	A45L	116	E77R
26	V93Y	117	R156A
27	V93G	118	K72M
28	L46A	119	S205A
29	L46H	120	Q209S
30	G98A	121	V212E

31	P20S	122	R213W
32	V93A	123	I216T
33	V103T	124	P217H
34	P108F	125	P217V
35	V93L	126	D219F
36	S101S	127	E220V
37	A106G	128	R221E
38	S101Q	129	F223C
39	P108T	130	S226P
40	N118G	131	L228F
41	P108C	132	V234A
42	I120L	133	S238S
43	A106W	134	V236T
44	N118R	135	V236T
45	N110A; N118G	136	T241R
46	N118A	137	L242F
47	N118R	138	T241R
48	P117W; N118K	139	T241C
49	D133N	140	E248F
50	K126Q	141	D250E
51	K126R	142	K257V
52	K128S	143	G256K
53	I127M	144	E260G
54	T131T	145	L262R
55	D133L	146	K263M
56	M132A	147	D267G
57	D133E	148	D267R
58	L129V	149	I265L
59	K126K	150	E268S
60	I130M	151	L270S
61	M132Y	152	L270G
62	K128R	153	L270W
63	M132R	154	R271S
64	L129I	155	I274W
65	K128L; D2D (GAC → GAT)	156	G278S
66	F137W	157	Y279C
67	I152V	158	S284R
68	N55L	159	E282G
69	N150S	160	T280N
70	L138L	161	V286G
71	P149P	162	R285F
72	G161G	163	V286R
73	A165T	164	G240G
74	H163R	165	E61R
75	H163K	166	E61D
76	H168A	167	E61Y
77	E171S	168	G85G
78	E171R	169	G85D
79	E171R	170	S80R
80	T172I	171	Y79R

81	C176G	172	Y79V
82	A177S	173	W283V
83	A177S	174	W283E
84	S80L; R156W	175	W283A
85	H182G	176	W283S
86	N186S	177	W283G
87	K185R	178	W283A
88	K185T	179	W283R
89	D2H	180	W283T
90	D2T; E260G	181	P281W
91	D2Y	182*	V236T; T241R

*Mutant 182 was created using site-directed mutagenesis, using Mutant 136 DNA as a template and then introducing the V236T mutation. One skilled in the art can synthesize this gene using site-directed mutagenesis techniques.

Samples listed in Table 43 were then prepared for the tertiary screen. Glycerol stocks were used to inoculate 5 mL of LB containing 100 μg/mL carbenicillin. Cultures were grown overnight at 37°C. The overnight cultures were then used to inoculate 50 mL cultures of LB containing 100 μg/mL carbenicillin in 250 mL baffled flasks to OD_{600nm} of 0.05. IPTG was added to a final concentration of 1 mM when the OD_{600nm} reached 0.4 - 0.8. Cultures were induced overnight at 30°C. Cell pellets were harvested by centrifugation at 6,000 rpm for 20 minutes. Cell pellets were frozen at -20°C until cell lysates could be prepared. Cells were lysed with BPER II (Thermo Scientific, Rockford, IL) on ice for 1 hour. Clarified lysates were prepared by centrifugation at 12,000 rpm for 30 minutes.

Protein was quantified by Bio-Rad Bradford Protein Assay (Bio-Rad, Hercules, CA) per the manufacturer's instructions. SDS-PAGE analysis and densitometry were used to determine the amount of expressed D-aminotransferase. Samples were normalized for expressed D-aminotransferase. 0.02 mg/mL D-aminotransferase was tested in the tertiary screen. The tertiary screening method was the same as the secondary screening method except that samples were taken at 0, 5, 10, 15, 30, 60, 120 and 210 minutes to develop a timecourse. Alanine production and monatin consumption values were measured by LC/MS/MS analysis and compared to a standard curve. Samples were compared to the wild type control.

Samples with higher final titers or faster initial rates than the wild type control were identified as hits and are referred to as upmutants. The GSSM^{SM} upmutants identified in the tertiary screen are listed in Table 44. These upmutants are further described in Example 27 below.

Example 27—Enzymatic Activity of Polypeptides Upmutants

This example describes data demonstrating the enzymatic activity of exemplary upmutant polypeptides disclosed herein, e.g., the polypeptides having amino acid sequences described in Table 44. Table 44 shows the activity of the upmutants relative to the wild type control at the 15 minute time point in reactions using 1 mM and 15 mM R,R-Monatin substrate. Relative activity is the amount of alanine produced by the sample divided by the amount of alanine produced by the wild type control.

Table 44: Activity of GSSM Upmutants in Tertiary Screen

Mutant	Mutation	Activity relative to wild type control (SEQ ID NO:220)	
		Reaction with 1 mM monatin substrate	Reaction with 15 mM monatin substrate
23	V42M	1.28	1.04
24	G41Y	1.37	1.31
27	V93G	1.73	1.98
31	P20S	1.29	1.60
35	V93L	1.14	0.96
40	N118G	2.61	1.52
44	N118R	1.55	0.47
45	N110A; N118G	2.50	2.02
46	N118A	2.28	0.69
48	P117W; N118K	2.54	1.12
58	L129V	1.04	0.85
66	F137W	1.25	1.44
67	I152V	1.19	1.24
81	C176G	1.11	1.27
82	A177S	1.24	1.02
104	I64H	1.37	1.07
109	T74L	1.37	1.31
110	K73L	2.83	3.75
111	T74V	1.99	2.19
112	T74M	1.78	2.01
135	V236T	3.44	2.88
136	T241R	2.64	1.79
152	L270G	1.24	0.89
153	L270W	2.00	1.54
174	W283E	1.23	0.84
175	W283A	1.61	1.09
177	W283G	1.71	1.06
6	Y6L	2.52	2.21
88	K185T	1.04	0.95
107	L66G	1.08	1.02

Several samples were identified that outperformed the wild type control under the conditions tested. Potential K_m and V_{max} upmutants were identified. These results indicate that the wild type control (SEQ ID NO:220) is further evolvable for increased specific D-aminotransferase activity on monatin.

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Example 28—Activity of GSSM^{SM} Mutants in Monatin Process

Analysis of GSSM DATs in pSE420-C-His

This example describes data demonstrating the enzymatic activity of exemplary polypeptides disclosed herein. Mutant 27, Mutant 44, Mutant 58, Mutant 119, Mutant 135, Mutant 136, Mutant 152, Mutant 154 and the wild type control (in vector pSE420-C-His in *E. coli* XL1-Blue, as described in Examples 25 and 26) were streaked onto agar plates containing LB medium with ampicillin (100 μg/mL). Single colonies were used to inoculate 5 mL of LB medium containing ampicillin (100 μg/mL). Five hundred μl were used to inoculate 50 mL of the same medium in a 250 mL baffled flask. The cells were grown at 30°C to approximately an OD_{600nm} of 0.4. IPTG was added to a final concentration of 1 mM. Cells were grown at 30°C for 4 hours and collected by centrifugation. Cells were immediately frozen at –80°C until cell extracts were prepared.

Cell extracts were prepared as described in Example 4. Protein concentrations were determined using the BCA (Pierce, Rockford, IL) microtiter plate assay with BSA (Pierce Rockford, IL) as the standard, per the manufacturer's instructions. To estimate the concentration of the D-aminotransferase in the cell-free extracts, SDS-PAGE analysis was done and visual estimation was used to estimate percentage of expression. The DAT proteins were soluble in the range of 10-25% expression as percentage of total protein and this was used to calculate the dosage of the assays.

An R,R monatin formation assay was performed containing 100 mM EPPS buffer pH 7.8, 1 mM $MgCl_2$, 0.05 mM PLP, 200 mM sodium pyruvate, 10 mM potassium phosphate, 0.01% Tween-80 with 0.1 mg/mL aldolase and 0.2 mg/mL of DAT in a 4 mL reaction at room temperature. Mutant 27 used 0.15 mg/mL of DAT enzyme instead of 0.2 mg/mL. After 0.5, 1, 2, 4 and 23 hours, an aliquot was taken, formic acid was added to a final concentration of 2%, and the samples spun and filtered. Samples were analyzed for monatin using the LC/MS/MS methodology described in Example 36. Results are shown in Table 45.

Table 45: Activity of DATs (cloned into pSE420-C-His)

DAT polypeptide	Monatin (mM) 0.5 hr	Monatin (mM) 1 hr	Monatin (mM) 2 hr	Monatin (mM) 4 hr

wild type control	2.12	5.26	9.34	13.05
Mutant 27	4.74	9.55	14.72	18.06
Mutant 44	3.73	6.61	10.38	13.23
Mutant 58	3.61	7.51	11.85	14.56
Mutant 135	3.50	7.72	12.50	16.17
Mutant 136	1.40	4.63	6.59	--
Mutant 152	4.79	9.19	13.08	14.85
Mutant 154	3.76	7.66	11.85	14.38

As can be seen from the data shown in Table 45, a number of DAT mutants obtained through GSSM^{SM} evolution showed improved initial rates of monatin formation over the wild type control under the conditions of the assay.

Analysis of GSSM^{SM} DATs in pMet1a

This example describes data demonstrating the enzymatic activity of exemplary polypeptides disclosed herein. Mutant 2, Mutant 6, Mutant 11, Mutant 27, Mutant 40, Mutant 44, Mutant 45, Mutant 58, Mutant 110, Mutant 135, and Mutant 136 were recreated by site directed mutagenesis using QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. To generate the mutants, the pMET1a tagged construct described in Example 16 (pMET1a:SEQ ID NO:220(WT)) was used as the template. The mutagenic primers used are listed below in Table 46. The PCR fragments were digested with *Dpn*1 (Invitrogen, Carlsbad, CA) for 1 hour and transformed into *E. coli* Top10 cells (Invitrogen, Carlsbad, CA). The resultant purified plasmid preparations were sequenced (Agencourt, Beverly, MA) to verify that the correct mutations were incorporated. The plasmids were then transformed into *E. coli* B834(DE3) expression host (Novagen, San Diego, CA).

Table 46: Primers for Mutagenesis

Mutant produced	PCR primers	Template
Mutant 2	5'-ATG GAC GCA CTG GGA TGT TAC AAC GGA AAT TGG -3' (SEQ ID NO:1084) 5'- CCA ATT TCC GTT GTA ACA TCC CAG TGC GTC CAT -3' (SEQ ID NO:1085)	SEQ ID NO:220
Mutant 6	5'- ATG GAC GCA CTG GGA CTT TAC AAC GGA AAT TGG GGG-3' (SEQ ID NO:1086) 5'- CCC CCA ATT TCC GTT GTA AAG TCC CAG TGC GTC CAT-3' (SEQ ID NO:1087)	SEQ ID NO:220
Mutant 27	5'- TAC CTG GTT TAT TGG CAG GGT ACT CGC GGA ACA GGC CGG-3' (SEQ ID NO:1088) 5'- CCG GCC TGT TCC GCG AGT ACC CTG CCA ATA AAC CAG GTA-3' (SEQ ID NO:1089)	SEQ ID NO:220
Mutant	5'- CTC TGG ATT ATA ATT AAG CCC GGC CAC ATC GAC	SEQ ID

40	AAT CTT TAT AG -3' (SEQ ID NO:1090) 5'- CTA TAA AGA TTG TCG ATG TGG CCG GGC TTA ATT ATA ATC CAG AG -3' (SEQ ID NO:1091)	NO:220
Mutant 44	5'- CTC TGG ATT ATA ATT AAG CCC AGG CAC ATC GAC AAT CTT TAT AG-3' (SEQ ID NO:1092) 5'- CTA TAA AGA TTG TCG ATG TGC CTG GGC TTA ATT ATA ATC CAG AG-3' (SEQ ID NO:1093)	SEQ ID NO:220
Mutant 45	5'- GTA TTT CCG GCA GGC CCT TCA GCG CTC TGG ATT ATA ATT AAG CC -3' (SEQ ID NO:1094) 5'- GGC TTA ATT ATA ATC CAG AGC GCT GAA GGG CCT GCC GGA AAT AC -3' (SEQ ID NO:1095)	Mutant 40
Mutant 58	5'- CAA TCT TTA TAG AAA AAT CAA GGT TAT TAC CAT GGA TGA TAC CCG C 3' (SEQ ID NO:1096) 5'- GCG GGT ATG ATC CAT GGT AAT AAC CTT GAT TTT TCT ATA AAG ATT G -3' (SEQ ID NO:1097)	SEQ ID NO:220
Mutant 110	5'- CTT AAC AAA AGA GGA ATT GAA ACT GAC TTT AAA TGA AAT GTA CTC C-3' (SEQ ID NO:1098) 5'- GGA GTA CAT TTC ATT TAA AGT CAG TTT CAA TTC CTC TTT TGT TAA G -3' (SEQ ID NO:1099)	SEQ ID NO:220
Mutant 135	5'- TTC GAC GCG GAC GAG GTG CTT ACT TCC AGC AGC GGC ACA CTC G-3' (SEQ ID NO:1100) 5'- CGA GTG TGC CGC TGC TGG AAG TAA GCA CCT CGT CCG CGT CGA A-3' (SEQ ID NO:1101)	SEQ ID NO:220
Mutant 136	5'- TGC TTG TGT CCA GCA GCG GCC GGC TCG GCC TTA GCG CCG-3' (SEQ ID NO:1102) 5'- CGG CGC TAA GGC CGA GCC GGC CGC TGC TGG ACA CAA GCA-3' (SEQ ID NO:1103)	SEQ ID NO:220

Mutant 2, Mutant 6, Mutant 27, Mutant 40, Mutant 45, Mutant 58, Mutant 110, Mutant 119, Mutant 131, Mutant 135, Mutant 136, Mutant 152, Mutant 154 were generated in the pMET1a vector and transformed into the compatible *E. coli* expression host B834(DE3) (Novagen, San Diego, Ca) described in Example 2. Overnight cultures in LB medium containing carbenicillin (100 μg/mL) were diluted 1:100 in 100 mL of the same medium and grown in a 500 mL baffled flask. The culture was grown at 30°C overnight to an OD_{600nm} of 10 in Overnight Express II medium (Solution 1-6, Novagen). Samples for total protein were taken immediately prior to harvesting. Cells were harvested by centrifugation and washed once with 10 mL of potassium phosphate buffer pH 7.8. Cells were immediately frozen at –80°C until cell extracts were prepared. It is noted that, in addition to site-directed mutagenesis, one skilled in the art can synthesize the genes encoding these D-aminotransferases using multi-change mutagenesis PCR techniques such as those described in Example 25.

Cell extracts were prepared and desalted as described in Example 4 using 100 mM potassium phosphate as the buffer to elute and equilibrate the PD10 column. Total protein and DAT concentrations were determined as described.

Transamination of R,R monatin with pyruvate as the amino acceptor were performed as described in Example 5 except that 15 mM R,R monatin was utilized. Initial analyses of alanine, monatin, and monatin precursor levels identified Mutant 40, Mutant 135 and Mutant 136 as superior mutants resulting in the highest levels of alanine production as shown in Table 47. DAT Mutant 136 appeared to have the highest activity for conversion of R,R monatin to R-MP. The alanine production numbers (in mM) for the various time points are shown in Table 47.

Table 47: Alanine formation (mM) from R,R monatin transamination reactions from DATs cloned into pMET1a

DAT polypeptide	Alanine (mM) 15 minutes	Alanine (mM) 30 minutes	Alanine (mM) 60 minutes	Alanine (mM) 120 minutes
wild type control	3.08	5.47	8.19	10.07
Mutant 2	3.38	5.74	8.85	10.52
Mutant 6	3.51	5.97	8.99	10.81
Mutant 27	4.36	8.00	10.72	10.52
Mutant 40	7.89	10.37	11.79	12.50
Mutant 44	2.65	4.58	7.18	--
Mutant 58	3.90	6.95	9.93	10.52
Mutant 110	3.50	6.17	9.53	10.52
Mutant 135	5.35	8.64	10.82	10.91
Mutant 136	6.24	9.46	11.24	11.15
Mutant 152	4.26	7.12	9.83	10.32
Mutant 154	4.16	7.13	10.07	10.76

--: not determined under present conditions

To further assess activity, a monatin formation assay was done as described in Example 1 with a DAT concentration of approximately 0.2 mg/mL. As a control, 0.2 mg/mL concentration of purified wild type DAT was evaluated. After 0.5, 1, 2, and 4 hrs, an aliquot was taken and formic acid was added to a final concentration of 2%, and the samples were spun and filtered. Samples were analyzed for monatin using the LC/MS/MS methodology described herein and for tryptophan and alanine using the LC/OPA post-column fluorescence methodology described in Example 36.

Table 48: Activity of DATs in pMET1a

DAT polypeptide	Monatin (mM) 0.50 hr	Monatin (mM) 1.00 hr	Monatin (mM) 2.00 hr	Monatin (mM) 4.00 hr
wild type control	3.96	7.83	9.70	11.18
Mutant 2	1.56	3.78	8.77	12.68
Mutant 27	4.70	9.70	n.d.	13.80
Mutant 44	3.03	5.61	8.50	12.28
Mutant 45	1.40	4.00	7.70	11.50

Mutant 58	3.83	7.23	11.33	14.12
Mutant 110	2.60	5.90	9.90	12.70
Mutant 119	4.12	7.87	11.37	13.50
Mutant 131	3.75	7.41	11.40	13.90
Mutant 135	6.39	10.65	13.49	13.15
Mutant 136	3.36	8.02	12.86	13.16
Mutant 154	3.00	6.06	10.67	13.17

All the DATs shown in Table 48 produced monatin. DAT mutants Mutant 58, Mutant 135 and Mutant 136 had faster initial rates than the wild type control. Mutant 136 was slower for reaction one (conversion of D-Trp to I3P) but had better overall monatin production than the wild type control.

For the final time point, an additional aliquot was taken (without the addition of formic acid) to determine the stereoisomeric distribution of the monatin produced using the FDAA derivatization methodology described in Example 36. For the select mutants tested, there was little to no impact on stereopurity. In all cases, the mutants produced over 98.8% R,R under the assay conditions tested. These results are shown in Table 49.

Table 49: Stereopurities of Monatin Produced by Select Mutants at 4 hours

DAT polypeptide	%SS	%RS	%RR	%SR
wild type (pMet1a) control	0.00	0.40	99.30	0.20
Mutant 6	0.00	0.40	99.50	0.10
Mutant 27	0.00	0.80	98.80	0.30
Mutant 40	0.00	0.20	99.80	0.00
Mutant 45	0.00	0.50	99.40	0.10
Mutant 110	0.10	0.40	99.30	0.10
Mutant 135	0.00	0.40	99.50	0.10
Mutant 136	0.02	1.00	99.00	0.03

Example 29—Construction and Testing of Tailored Multi-Site Combinatorial Assembly (TMCA^{SM}) Mutants

This example describes the construction of exemplary nucleic acids and polypeptides, and describes their enzymatic activity. A subset of GSSM mutations were selected for combination using Tailored Multi-Site Combinatorial Assembly^{SM} (TMCA^{SM}) technology. The top ten performers from the GSSM evolution in either the 1 or 15 mM monatin reactions were selected for TMCA^{SM} evolution. The wild type sequence (SEQ ID NO:220) was threaded onto a model of 3DAA-D amino acid aminotransferase (Figure 4). The model in Figure 4 is shown with pyridoxyl-5'-phosphate D-alanine, with the numbered residues indicating those sites selected for TMCA^{SM} evolution. Table 50 also lists the mutations that were selected for

inclusion in the TMCA^{SM} library. TMCA^{SM} evolution was performed on wild type (SEQ ID NO:220) and Mutant 45 using the methods described in PCT Application No. PCT/US08/071771.

TMCA evolution is described in PCT Application Number PCT/US08/071771 and comprises a method for producing a plurality of progeny polynucleotides having different combinations of various mutations at multiple sites. The method can be performed, in part, by a combination of at least one or more of the following steps:

Obtaining sequence information of a ("first" or "template") polynucleotide. For example, the first or template sequence can be a wild type (e.g. SEQ ID NO:220) or mutated (e.g. Mutant 45) sequence. The sequence information can be of the complete polynucleotide (e.g., a gene or an open reading frame) or of partial regions of interest, such as a sequence encoding a site for binding, binding-specificity, catalysis, or substrate-specificity.

Identifying three or more mutations of interest along the first or template polynucleotide sequence. For example, mutations can be at 3, 4, 5, 6, 8, 10, 12, 20 or more positions within the first or template sequence. The positions can be predetermined by absolute position or by the context of surrounding residues or homology. For TMCA of DAT polypeptides, the top 10 codon changes that resulted in improved enzyme performance were included as mutations of interest. The sequences flanking the mutation positions on either side can be known. Each mutation position may contain two or more mutations, such as for different amino acids. Such mutations can be identified by using Gene Site Saturation Mutagenesis^{SM} (GSSM ^{SM}) technology, as described herein and in U.S. Patent Nos. 6,171,820; 6,562,594; and 6,764,835.

Providing primers (e.g., synthetic oligonucleotides) comprising the mutations of interest. In one embodiment, a primer is provided for each mutation of interest. Thus, a first or template polynucleotide having 3 mutations of interest can use 3 primers at that position. The primer also can be provided as a pool of primers containing a degenerate position so that the mutation of interest is the range of any nucleotide or naturally occurring amino acid, or a subset of that range. For example, a pool of primers can be provided that favor mutations for aliphatic amino acid residues.

The primers can be prepared as forward or reverse primers, or the primers can be prepared as at least one forward primer and at least one reverse primer. When mutations are positioned closely together, it can be convenient to use primers that contain mutations for more than one position or different combinations of mutations at multiple positions.

Providing a polynucleotide containing the template sequence. The first or template polynucleotide can be circular, or can be supercoiled, such as a plasmid or vector for cloning, sequencing or expression. The polynucleotide may be single-stranded ("ssDNA"), or can be

double-stranded ("dsDNA"). For example, the TCMA method subjects the supercoiled ("sc") dsDNA template to a heating step at 95°C for 1 min (see Levy, *Nucleic Acid Res.*, 28(12):e57(i-vii) (2000)).

Adding the primers to the template polynucleotide in a reaction mixture. The primers and the template polynucleotide are combined under conditions that allow the primers to anneal to the template polynucleotide. In one embodiment of the TMCA protocol, the primers are added to the polynucleotide in a single reaction mixture, but can be added in multiple reactions.

Performing a polymerase extension reaction. The extension products (e.g., as a "progeny" or "modified extended polynucleotide") may be amplified by conventional means. The products may be analyzed for length, sequence, desired nucleic acid properties, or expressed as polypeptides. Other analysis methods include *in-situ* hybridization, sequence screening or expression screening. The analysis can include one or more rounds of screening and selecting for a desired property.

The products can also be transformed into a cell or other expression system, such as a cell-free system. The cell-free system may contain enzymes related to DNA replication, repair, recombination, transcription, or for translation. Exemplary hosts include bacterial, yeast, plant and animal cells and cell lines, and include *E. coli*, *Pseudomonas fluorescens*, *Pichia pastoris* and *Aspergillus niger*. For example, XL1-Blue or Stb12 strains of *E. coli* can be used as hosts.

The method of the invention may be used with the same or different primers under different reaction conditions to promote products having different combinations or numbers of mutations.

By performing the exemplary method described above, this protocol also provides one or more polynucleotides produced by this TMCA evolution method, which then can be screened or selected for a desired property. One or more of the progeny polynucleotides can be expressed as polypeptides, and optionally screened or selected for a desired property. Thus, this embodiment of the TMCA evolution protocol provides polynucleotides and the encoded polypeptides, as well as libraries of such polynucleotides encoding such polypeptides. This embodiment of the TMCA evolution protocol further provides for screening the libraries by screening or selecting the library to obtain one or more polynucleotides encoding one or more polypeptides having the desired activity.

Another embodiment of the TMCA evolution protocol described in PCT/US08/071771 comprises a method of producing a plurality of modified polynucleotides. Such methods generally include (a) adding at least three primers to a double stranded template polynucleotide in a single reaction mixture, wherein the at least three primers are not overlapping, and wherein each of the at least three primers comprise at least one mutation different from the other primers,

wherein at least one primer is a forward primer that can anneal to a minus strand of the template and at least one primer is a reverse primer that can anneal to a plus strand of the template, and (b) subjecting the reaction mixture to a polymerase extension reaction to yield a plurality of extended modified polynucleotides from the at least three primers.

Another embodiment of the TMCA evolution protocol described in PCT/US08/071771 comprises a method wherein a cell is transformed with the plurality of extended products that have not been treated with a ligase. In another embodiment of the invention, the plurality of extended modified polynucleotides is recovered from the cell. In another embodiment, the recovered plurality of extended modified polynucleotides is analyzed, for example, by expressing at least one of the plurality of extended modified polynucleotides and analyzing the polypeptide expressed therefrom. In another embodiment, the plurality of extended modified polynucleotides comprising the mutations of interest is selected.

In another embodiment of the TMCA evolution protocol, sequence information regarding the template polynucleotide is obtained, and three or more mutations of interest along the template polynucleotide can be identified. In another embodiment, products obtained by the polymerase extension can be analyzed before transforming the plurality of extended modified products into a cell.

In one embodiment of the TMCA evolution protocol, products obtained by the polymerase extension are treated with an enzyme, e.g., a restriction enzyme, such as a *DpnI* restriction enzyme, thereby destroying the template polynucleotide sequence. The treated products can be transformed into a cell, e.g., an *E.coli* cell.

In one embodiment of the TMCA evolution protocol, at least two, or at least three, or at least four, or at least five, or at least six, or at least seven, or at least eight, or at least nine, or at least ten, or at least eleven, or at least twelve, or more primers can be used. In one embodiment, each primer comprises a single point mutation. In another embodiment, two forward or two reverse primers comprise a different change in the same position on the template polynucleotide. In another embodiment, at least one primer comprises at least two changes in different positions on the template polynucleotide. In yet another embodiment, at least one primer comprises at least two changes in different positions and at least two forward or two reverse primers comprise a different change in the same position on the template polynucleotide.

In one embodiment of the TMCA evolution protocol, the forward primers are grouped into a forward group and the reverse primers are grouped into a reverse group, and the primers in the forward group and the primers in the reverse group, independent of one another, are normalized to be equal concentration in the corresponding group regardless of positions on the template polynucleotide, and wherein after the normalization an equal amount of the forward and

reverse primers is added to the reaction. In this normalization method, a combination of some positions may be biased. The bias can be due to, for example, a relatively low primer concentration at one position containing a single primer compared to a position containing multiple primers. "Positional bias" refers to resulting polynucleotides which show a strong preference for the incorporation of primers at a single position relative to the other positions within its forward or reverse primer group. This results in a combination of modified polynucleotides which may have a high percentage of mutations within a single primer position but a low percentage of mutations at another position within its forward or reverse primer group. This bias is unfavorable when the goal of the TMCA is to generate progeny polynucleotides comprising all possible combinations of changes to the template. The bias can be corrected, for example, by normalizing the primers as a pool at each position to be equal.

In one embodiment of the TMCA evolution protocol, the primer normalization is performed by organizing the primers into multiple groups depending on their location on the template polynucleotide, wherein the primers covering the same selected region on the template are in one group; normalizing the grouped primers within each group to be equal concentration; pooling the forward primers within one group into a forward group and normalizing concentration between each group of the forward primers to be equal; pooling the reverse primers within one group into a reverse group and normalizing concentration between each group of the reverse primers to be equal; and adding an equal amount of the pooled forward and reversed primers into the reaction. No bias has been observed for position combinations.

In one embodiment of the TMCA evolution protocol, a set of degenerate primers each comprising a degenerate position is provided, wherein the mutation of interest is a range of different nucleotides at the degenerate position. In another embodiment, a set of degenerate primers is provided comprising at least one degenerate codon corresponding to at least one codon of the template polynucleotide and at least one adjacent sequence that is homologous to a sequence adjacent to the codon of the template polynucleotide sequence. In another embodiment, the degenerated codon is N,N,N and encodes any of 20 naturally occurring amino acids. In another embodiment, the degenerated codon encodes less than 20 naturally occurring amino acids.

Another embodiment of the TMCA evolution protocol described in PCT/US08/071771 comprises a method of producing a plurality of modified polynucleotides comprising the mutations of interest. Such methods generally include (a) adding at least two primers to a double stranded template polynucleotide in a single reaction mixture, wherein the at least two primers are not overlapping, and wherein each of the at least two primers comprise at least one mutation different from the other primer(s), wherein at least one primer is a forward primer that can anneal

to a minus strand of the template and at least one primer is a reverse primer that can anneal to a plus strand of the template, (b) subjecting the reaction mixture to a polymerase extension reaction to yield a plurality of extended modified polynucleotides from the at least two primers, (c) treating the plurality of extended modified polynucleotides with an enzyme, thereby destroying the template polynucleotide, (d) transforming the treated extended modified polynucleotides that have not been treated with a ligase into a cell, (e) recovering the plurality of extended modified polynucleotides from the cell, and (f) selecting the plurality of extended modified polynucleotides comprising the mutations of interest.

Table 50: List of Sites for TMCA evolution

Mutation	New Codon
P20S	AGT
K73L	TTG
T74V	GTG
V93G	GGT
N110A	GCT
P117W	TGG
N118G	GGG
N118A	GCG
V236T	ACT
T241R	CGG
L270W	TGG

TMCA mutants were grown, arrayed, assayed and sequenced using the same method as described for the GSSM evolution in Example 25. Sample performance was compared to the performance of the top candidate from GSSM evolution – Mutant 135 – using the same scoring system as described in Example 25. Table 52 lists the TMCA secondary screen hits with unique DNA sequences (TMCA mutants are designated with alphabetic characters to distinguish them from GSSM mutants, which are designated numerically).

Table 52: TMCA Mutants Identified as Secondary Screen Hits

Mutant name	Mutation	Mutant name	Mutation
A	P20S-N118G	EE	P20S-N110A-N118G
B	T74V-V93G-L270W	FF	N110A-N118G-T241R-L270W
C	P20S-T74V-L270W	GG	P20S-T74V-V93G-N110A-N118G-V236T-L270W
D	T74V-L270W	HH	V93G-N110A-N118G-V236T
E	P20S-K73L-T241R-L270W	II	P20S-T74V-N110A-N118G
F	K73L-V93G-V236T-T241R	JJ	N110A-N118G
G	P20S-T74V-V236T	KK	P20S-V93G-N110A-N118G-T241R

H	P20S-K73L-V93G	LL	N110A-N118A-L270W
I	K73L-V236T	MM	P20S-N110A-N118G-L270W
J	P20S-L270W	NN	N110A-N118A-V236T-T241R
K	2N-P20S-K73L-V93G-N118G	OO	N110A-N118G-L270W
L	P20S-T74V-N118A	PP	V93G-N110A-N118G-T241R
M	P20S-V236T	QQ	P20S-V93G-N110A-N118G
N	P20S-T241R-L270W	RR	V93G-N110A
O	P20S-T241R	SS	P20S-N110A-N118G-V236T
P	T74V-V93G-V236T-T241R	TT	T74V-N110A-N118A-V236T
Q	P20S-K73L-T74V-L270W	UU	P20S-K73L-T74V-N110A-N118G-V236T-T241R
R	P20S-V93G-V236T	VV	86E (SILENT GAG → GAA)-N110A-N118A-V236T
S	P20S-K73L-T74V-T241R-L270W	WW	T74V-N118G
T	P20S-K73L-L270W	XX	P20S-T241R-L270W-277T (SILENT ACA → ACG)
U	T74V-V93G-N118G-V236T-T241R	YY	T74V-N118A-L270W
V	P20S-K73L-210A (SILENT-GCC → GCT)-V236T	ZZ	P20S-K73L-N118A-L270W
W	N118A-L270W	AAA	P20S-V93G-T241R
X	P20S-58K (SILENT AAG → AAA)-L270W	BBB	T74V-V93G-N110A-T241R
Y	P20S-V93G-N118G	CCC	V93G-N110A-N118A
Z	P20S-V236T-T241R	DDD	P20S-T74V-V93G-N110A-N118G-T241R
AA	P20S-P117W-N118A-V236T-L270W	EEE	T74V-N110A-N118G-L270W
BB	V93G-V236T	FFF	P20S-231A (SILENT GCG → GCA)-V236T
CC	V236T-L270W	GGG	V93G-V236T-T241R
DD	P20S-N118G-L270W		

The samples identified in Table 52 were grown, normalized and assayed in the tertiary screen using the same method as described for the GSSM evolution in Example 26. Monatin and alanine values were determined by LC/MS/MS and compared to a standard curve. Sample performance was compared to the activity of Mutant 135 (the top performer from GSSM evolution). TMCA upmutants identified in the tertiary screen are listed in Table 53.

Example 31—Activity of TMCA Hits

This example describes data demonstrating the enzymatic activity of exemplary polypeptides. Table 53 below shows the activity of the upmutants relative to Mutant 135 at the 15 minute time point in reactions using 1 mM and 15 mM R,R-monatin substrate. Relative activity is the amount of alanine produced by the sample divided by the amount of alanine produced by Mutant 135.

Table 53: Activity of TMCA Upmutants in Tertiary Screen

Mutant	Mutation	Activity relative to GSSM Mutant 135	
		Reactions with 1 mM monatin substrate	Reactions with 15 mM monatin substrate
C	P20S-T74V-L270W	1.02	0.93
E	P20S-K73L-T241R-L270W	1.32	1.31
F	K73L-V93G-V236T-T241R	1.29	0.64
G	P20S-T74V-V236T	1.28	1.30
I	K73L-V236T	1.24	1.29
J	P20S-L270W	0.79	1.01
L	P20S-T74V-N118A	1.62	0.83
M	P20S-V236T	1.27	1.46
O	P20S-T241R	1.33	1.71
R	P20S-V93G-V236T	1.22	1.02
S	P20S-K73L-T74V-T241R-L270W	1.16	1.18
V	P20S-K73L-210A (SILENT-GCC → GCT)-V236T	1.03	1.00
Z	P20S-V236T-T241R	1.02	0.89
BB	V93G-V236T	1.55	1.98
CC	V236T-L270W	1.24	1.40
DD	P20S-N118G-L270W	1.54	1.78
PP	V93G-N110A-N118G-T241R	1.40	1.53
TT	T74V-N110A-N118A-V236T	1.10	0.42
VV	86E (SILENT GAG → GAA)-N110A-N118A-V236T	1.31	0.52
WW	T74V-N118G	1.23	1.49
YY	T74V-N118A-L270W	1.97	1.30
ZZ	P20S-K73L-N118A-L270W	1.01	0.44
AAA	P20S-V93G-T241R	1.86	3.49
CCC	V93G-N110A-N118A	1.26	0.56

Several samples were identified that outperformed Mutant 135 under the conditions tested. Potential K_m and V_{max} upmutants were identified. The results of the GSSM and TMCA evolutions indicate that wild type SEQ ID NO:220 is further evolvable for increased specific activity on monatin.

Example 32—Evaluation of TMCA Mutant DATs in pMET1a

This example describes data demonstrating the enzymatic activity of exemplary polypeptides disclosed herein. Mutant E, Mutant G, Mutant I, Mutant M, Mutant O, Mutant P, Mutant BB, Mutant PP, Mutant WW, and Mutant AAA (DATs created using TMCA technology, see Examples 29 and 30) were recreated by site-directed mutagenesis using QuikChange Multi

Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. To generate the mutants, pMET1a tagged constructs described in Example 16 and Example 28 were used as templates. The mutagenic primers used are listed below in Table 54. The PCR fragments were digested with *Dpn*1 (Invitrogen, Carlsbad, CA) for 1 hour and transformed into *E. coli* XL10-Gold cells (Stratagene, La Jolla, CA). The resultant purified plasmid preparations were sequenced (Agencourt, Beverly, MA) to verify that the correct mutations were incorporated. The plasmids were then transformed into *E. coli* B834(DE3) expression host (Novagen, San Diego, CA).

Table 54: Primers for Mutants in pMET1a Vector

TMCA mutant polypeptide produced	PCR primers	Template
Mutant E	5'- CTG GAC GAG ATG ACT GTG AGT ATG AAC GAC AGG GGC TGC TAC -3' (SEQ ID NO:1104) 5'- TGC TTG TGT CCA GCA GCG GCC GGC TCG GCC TTA GCG CCG-3' (SEQ ID NO:1105) 5'CTA AAA AAA ATC CAG GAT GAA GTG TGG AGG GAA TTT ATC GAA GCG ACA GG3' (SEQ ID NO:1106)	Mutant 110
Mutant G	5'- CAA AAG AGG AAT TGA AAA AAG TGT TAA ATG AAA TGT ACT CC -3' (SEQ ID NO:1107) 5'- GGA GTA CAT TTC ATT TAA CAC TTT TTT CAA TTC CTC TTT TG -3' (SEQ ID NO:1108)	Mutant M
Mutant I	5'- CTT AAC AAA AGA GGA ATT GAA ACT GAC TTT AAA TGA AAT GTA CTC C-3' (SEQ ID NO:1109) 5'- GGA GTA CAT TTC ATT TAA AGT CAG TTT CAA TTC CTC TTT TGT TAA G -3' (SEQ ID NO:1110)	Mutant 135
Mutant M	5'- CTG GAC GAG ATG ACT GTG AGT ATG AAC GAC AGG GGC TGC TAC -3' (SEQ ID NO:1111) 5'- GTA GCA GCC CCT GTC GTT CAT ACT CAC AGT CAT CTC GTC CAG -3' (SEQ ID NO:1112)	Mutant 135
Mutant O	5'- CTG GAC GAG ATG ACT GTG AGT ATG AAC GAC AGG GGC TGC TAC -3' (SEQ ID NO:1113) 5'- GTA GCA GCC CCT GTC GTT CAT ACT CAC AGT CAT CTC GTC CAG -3' (SEQ ID NO:1114)	Mutant 136
Mutant P	5'- CAA AAG AGG AAT TGA AAA AAG TGT TAA ATG AAA TGT ACT CC -3' (SEQ ID NO:1115) 5'- TTC GAC GCG GAC GAG GTG CTT ACT TCC AGC AGC GGC ACA CTC G-3' (SEQ ID NO:1116) 5'- TGC TTG TGT CCA GCA GCG GCC GGC TCG GCC TTA GCG CCG-3' (SEQ ID NO:1117)	Mutant 27
Mutant BB	5'- TAC CTG GTT TAT TGG CAG GGT ACT CGC GGA ACA GGC CGG-3' (SEQ ID NO:1118) 5'- CCG GCC TGT TCC GCG AGT ACC CTG CCA ATA AAC CAG GTA-3' (SEQ ID NO:1119)	Mutant 135

Mutant PP	5'- TAC CTG GTT TAT TGG CAG GGT ACT CGC GGA ACA GGC CGG-3' (SEQ ID NO:1120) 5'- TGC TTG TGT CCA GCA GCG GCC GGC TCG GCC TTA GCG CCG-3' (SEQ ID NO:1121)	Mutant 45
Mutant WW	5'- CAA AAG AGG AAT TGA AAA AAG TGT TAA ATG AAA TGT ACT CC -3' (SEQ ID NO:1122) 5'- GGA GTA CAT TTC ATT TAA CAC TTT TTT CAA TTC CTC TTT TG -3' (SEQ ID NO:1123)	Mutant M
Mutant AAA	5'- CTG GAC GAG ATG ACT GTG AGT ATG AAC GAC AGG GGC TGC TAC -3' (SEQ ID NO:1124) 5'- TGC TTG TGT CCA GCA GCG GCC GGC TCG GCC TTA GCG CCG-3' (SEQ ID NO:1125)	Mutant 27

E. coli B834(DE3) (Novagen, San Diego, CA) cultures expressing carboxy-terminal His-tagged Mutant 110, Mutant 135, Mutant 136, Mutant E, Mutant G, Mutant I, Mutant M, Mutant O, Mutant P, Mutant BB, Mutant PP, Mutant WW, Mutant AAA and wild type (SEQ ID NO:220) proteins were grown in 200 mL of Overnight Express II medium (Solution 1-6, Novagen) in a 500 mL baffled flask overnight at 30°C to an OD_{600} of 10. Samples for total protein were taken immediately prior to harvesting. Cells were harvested by centrifugation and immediately frozen at –80°C until cell extracts were prepared as described in Example 4.

Cell extracts were created by the addition of 50 mL of Bug Buster Primary Amine Free (Novagen,San Diego, CA) with 50 µl of Benzonase Nuclease (Novagen,San Diego, CA), 0.75 µl of rLysozyme (Novagen, San Diego, CA), and 250 µl of Protease Inhibitor Cocktail II (Calbiochem, San Diego, CA). The cells were incubated for 15 minutes at room temperature with gentle rocking. The extracts were centrifuged at 45,000 x g for 10 minutes.

His-tagged proteins were purified as described in Example 4 using GE Healthcare (Piscataway, NJ) Chelating Sepharose™ Fast Flow resin. The exception was Mutant 182, which was analyzed as CFE as described in Example 4. Purified protein was desalted using a PD10 column into 100 mM potassium phosphate, pH 7.8 with 0.050 mM PLP. Total protein and DAT concentrations were determined as described in Example 4.

A 3-step monatin formation assay was done as described in Example 5 with a DAT concentration of approximately 0.2 mg/mL and the aldolase at a concentration of 0.1 mg/mL. As a control, 0.2 mg/mL concentration of purified wild type DAT (SEQ ID NO:220) was evaluated. After 0.5, 1, 2, 4 and 24 hours, an aliquot was taken, formic acid was added to a final concentration of 2% and the samples were spun and filtered. Samples were analyzed for monatin using LC/MS/MS methodology and for tryptophan and alanine using the LC/OPA post-column fluorescence methodology described in Example 36. At the last time point, an additional aliquot was taken (without pH adjustment) to determine %R,R monatin by the FDAA-derivatization method described in Example 36. The amount of monatin (mM) produced at

various time points can be found in Table 55. Stereopurity was also determined and the percent of the R,R stereoisomer can be found in the far right hand column. The stereoisomer R,S made up the majority of the balance.

Table 55: Activity of Select DAT Mutants

DAT polypeptide	Monatin (mM) 0.25 hr	Monatin (mM) 0.5 hr	Monatin (mM) 1 hr	Monatin (mM) 4 hr	%RR
Wild type control (SEQ ID NO:220)	1.60 (±0.42)	2.95 (±0.64)	5.00 (±0.85)	11.40 (±0.42)	99.50 (±0.08)
Mutant 110	1.70 (±0.00)	3.20 (±0.85)	5.75 (±0.21)	12.60 (±0.14)	99.48 (±0.32)
Mutant 135	3.65 (±0.35)	6.17 (±0.65)	10.33 (±0.32)	13.20 (±0.56)	99.42 (±0.11)
Mutant 136	2.60	5.00	8.10	12.90	98.98
Mutant 182	--	3.20	6.80	14.30	99.50
Mutant E	1.80	3.80	8.60	18.60	99.45
Mutant G	3.10	6.50	9.90	12.90	99.05
Mutant I	2.90	5.30	8.50	12.90	99.46
Mutant M	4.20	8.10	11.20	13.80	98.96
Mutant O	2.60	5.70	9.50	14.00	98.59
Mutant BB	4.20	8.20	11.40	13.70	98.97
Mutant PP	2.40	3.20	6.20	17.40	97.25
Mutant AAA	2.80	6.80	11.80	14.80	97.98

-- = not determined under conditions tested

The relative rates of monatin production under the conditions tested indicate the greatest improvement in initial activity from Mutant 135, Mutant 136, Mutant E, Mutant G, Mutant M, Mutant O, Mutant BB, and Mutant AAA as determined by comparing the rate of monatin formation with purified protein over the first hour between the mutants and the wild type control (SEQ ID NO:220) DAT. DATs Mutant E and Mutant AAA had high activity but were not well expressed (less than 5% of the total protein) nor very soluble under the conditions tested.

The assay samples were also analyzed for intermediates such as monatin precursor, I3P, and byproduct 4-hydroxy-4-methyl glutmatic acid (HMG) as described in Example 36. The analysis of the amount of HMG formed was determined for the mutants Mutant E, Mutant G, Mutant I, Mutant M, Mutant O, Mutant BB, Mutant PP, Mutant AAA and Mutant 110, Mutant 135, and Mutant 136. It appears that at the 4 hour time point, more HMG were formed by the mutants Mutant 135, Mutant G, Mutant I, Mutant M and Mutant BB. These mutants all contained the change V236T. HMG was also present above the levels of the wild type control

(SEQ ID NO:220) with mutants Mutant E, Mutant G, Mutant M and Mutant AAA likely due to the change in residue P20S.

Table 56: HMG Formation by DAT Mutants after 4 hours

DAT polypeptide	HMG (mM) 4 hr
Wild type control (SEQ ID NO:220)	nd
Mutant 110	nd
Mutant 135	1.0
Mutant 136	nd
Mutant E	0.2
Mutant G	1.6
Mutant I	0.8
Mutant M	1.6
Mutant O	nd
Mutant BB	1.5
Mutant AAA	0.6

nd=not detected

DAT assay monitoring I3P formation

The formation of I3P from tryptophan was detected and monitored at a wavelength of 340 nm. Reactions were carried out in 1 mL reaction volume containing 900 μL of a 25 mM D-tryptophan, 25 mM pyruvic acid sodium salt, 0.05 mM PLP, 100 mM potassium phosphate (pH 7.8) solution combined with 100 μL dilutions of DAT (total protein) prepared as described above. Enzymes were diluted 1:100 and 1:200 with cold 50 mM potassium phosphate (pH 7.8) and 50 μM PLP prior to addition to the assay. Enzyme was added to the reaction mixture 1:100 and monitored in increments of 15 seconds for 3 minutes. The formation of indole-3-pyruvate (I3P) was monitored at a wavelength of 340 nm for 3 minutes on a BioRad Spectrophotometer (GE Healthscience, Piscataway, NJ) and rates were measured within the dynamic range of a standard curve. The standard curve was generated with purified wild type (SEQ ID NO:220) DAT protein and the concentration of DAT in cell extract was determined based on the equation of the line for the standard curve. The effective concentration of DAT with respect to the wild type DAT for the first reaction is reported in Table 57.

Table 57: Activity of DAT (Conversion of Tryptophan to I3P)

DAT Polypeptide	Rate of 13P formation (Δ Abs340 nm / minute)	Concentration of DAT (determined by activity)	Activity relative to wild type for first reaction

		mg/mL	
Wild type control (SEQ ID NO:220)	0.058	0.065	1.0
Mutant 135	0.067	0.075	1.2
Mutant 136	0.017	0.019	0.3
Mutant E	0.000	0.002	0.1
Mutant G	0.027	0.030	0.5
Mutant M	0.050	0.055	0.9
Mutant O	0.031	0.033	0.8
Mutant BB	0.045	0.050	0.8
Mutant AAA	0.002	0.004	0.2

The wild type DAT (SEQ ID NO:220) and mutants 136, E, G, M, O, BB and AAA can facilitate the conversion of both tryptophan to I3P and of monatin precursor to monatin. Table 57 shows that these mutants had lower activity for the conversion of tryptophan to I3P relative to the wild type DAT (SEQ ID NO:220). Yet, according to Table 55, the same mutants produced more total monatin from tryptophan than did the wild type DAT (SEQ ID NO:220). Thus, under the conditions of the assay described herein, there appears to be a beneficial effect on monatin production through controlling the conversion of tryptophan to I3P in the monatin biosynthetic pathway. For example, although Mutant E showed the lowest relative activity for conversion of tryptophan to I3P (see Table 57), it also produced the highest amount of monatin at 4 hours (see Table 55). Without being bound by theory, the beneficial effects of controlling the first step in the reaction could be attributed to a reduction of I3P buildup and subsequent potential I3P degradation to products other than monatin. Generally, it also appears that controlling the rate of one or more of the reactions involved in the production of monatin from tryptophan, using, for example, one or more mutant DATs, can have a beneficial effect on the total amount of monatin produced.

Example 33—Evaluation of Mutant DATs at 35°C

This example describes data demonstrating the enzymatic activity of exemplary polypeptides disclosed herein. Starter cultures were grown overnight at 37°C with shaking at 250 rpm until the OD_{600nm} reached 0.05. 200 mL of Overnight Express II medium (Novagen, San Diego, CA) was inoculated and grown as described in Example 3. Cultures were grown in duplicate and the cell pellets were combined. The pellets were resuspended in 40 mL of 50 mM sodium phosphate buffer (pH 7.8) with 0.05 mM PLP and lysed using a French Press (Sim Aminco, Rochester, NY) per the manufacturer's instructions. The supernatant was collected in a clean tube and stored at -80°C until used.

A 3-step monatin formation assay was performed as described in the methods with a DAT concentration of approximately 0.2 mg/mL and the aldolase at a concentration of 0.1 mg/mL in glass vials. Duplicate samples were incubated at either 25°C or 35°C and after 1, 3, and 4 hours, an aliquot was taken and formic acid was added to a final concentration of 2%, and the samples were spun and filtered. Samples were analyzed for monatin using LC/MS/MS methodology and for tryptophan and alanine using the LC/OPA post-column fluorescence methodology described in Example 36. Samples were also analyzed for intermediates such as monatin precursor, I3P, and 4-hydroxy-4-methyl glutmatic acid (HMG) as described in Example 36. The amount of monatin (mM) produced at various time points is shown in Table 58.

The monatin formation assay was repeated for the wild type control (SEQ ID NO:220), Mutant 135 and Mutant M under similar conditions except the reactions were carried out in plastic vials. Monatin production at various time points can be found in Table 58.

Table 58: Monatin Formation at 25°C and 35°C

DAT polypeptide	Monatin (mM) 1 hr	Monatin (mM) 3 hr	Monatin (mM) 4 hr
25°C			
wild type control (pMet1a)	2.0	6.8	8.4
Mutant 135 (V236T)	10.0	14.4	14.2
Mutant 136 (241R)	4.0	10.8	12.4
Mutant E (20S, 73L, 241R, 270W)	0.8	4.2	5.6
Mutant M (20S, 236T)	10.0	13.4	14.2
Mutant O (20S, 241R)	8.0	13.8	13.6
Mutant BB (93G, 236T)	4.0	11.4	12.4
Mutant AAA (20S, 93G, 241R)	0.2	1.0	1.6
35°C			
wild type control (pMet1a)	2.2	5.4	6.2
Mutant 135 (V236T)	9.4	9.2	9.8
Mutant 136 (241R)	4.8	9.4	10.4
Mutant E (20S, 73L, 241R, 270W)	0.6	3.4	4.2
Mutant M (20S, 236T)	9.2	13.6	14.6
Mutant O (20S, 241R)	9.6	10.6	10.8
Mutant BB (93G, 236T)	4.6	9.0	9.2
Mutant AAA (20S, 93G, 241R)	0.2	1.6	2.2

Lower monatin titers were observed using the DAT enzymes described here at 35°C under the conditions of the assay. However, select mutants Mutant 135, Mutant 136, Mutant M, Mutant O and Mutant BB showed increased initial monatin production rates and greater 4 hour monatin titers than the wild type control (SEQ ID NO:220) at 35°C under the assay conditions.

Example 34—Evaluation of Mutant DATs in BioReactors

This example describes data demonstrating the enzymatic activity of exemplary polypeptides disclosed herein in bioreactors. Glycerol stocks of the wild type control (SEQ ID NO:220), Mutant 135, Mutant 136, Mutant M, Mutant O, and Mutant BB were used to streak plates for single colonies. Single colonies were used to inoculate flasks containing 5 mL of LB medium with the appropriate antibiotic. The starter cultures were grown overnight at 37°C with shaking at 250 rpm and the OD_{600nm} was checked. When the OD_{600nm} reached 0.05, the 5 mL culture was inoculated into a 200 mL of Overnight Express II medium (Novagen, San Diego, CA) and then incubated at 30°C with shaking at 250 rpm. Each culture was grown in duplicate and the cell pellets were combined. Cultures were harvested by pelleting cells by centrifugation at 4000 rpm for 15 minutes. The supernatant was poured off and the pellet was either frozen for later use or resuspended in 40 mL of 50 mM sodium phosphate buffer (pH 7.8) and lysed using a French Press (Sim Aminco, Rochester, NY) or a microfluidizer (Microfluidics Corporation, Newton, MA) per the manufacturer's instructions. The supernatant was collected in a clean tube and stored at -80°C until used. Approximately 1 mL of the clarified lysate was retained for protein quantitation using the BCA assay (Pierce, Rockford, IL) and SDS-PAGE analysis.

Bench scale reactions (250 mL) were carried out in 0.7 L Sixfors agitated fermentors (Infors AG, Bottmingen, Switzerland) under a nitrogen headspace as described in Example 15. The reaction mix contained 10 mM potassium phosphate, 1 mM $MgCl_2$, 0.05 mM PLP, 200 mM sodium pyruvate and 130 mM D-tryptophan. The reaction mix was adjusted to 25°C and adjusted to pH 7.8 with potassium hydroxide. The aldolase described in Example 6 was added as a clarified cell extract at 0.02 mg/mL of target protein. Wild type control (SEQ ID NO:220), Mutant 135, Mutant 136, Mutant M, Mutant O, and Mutant BB DATs have soluble protein expressions ranging from 15-35% based on visual estimation. The clarified cell extracts were added at 0.20 mg/mL of target protein.

The progress of the reactions was followed by measuring monatin production at 1, 2, 4 and 24 hours using the LC/MS/MS methodology described in Example 36. The results are shown in Table 59.

Table 59: Monatin Production in Fermentors

DAT polypeptide	Protein Expression	Monatin (mM) 1 hr	Monatin (mM) 2 hr	Monatin (mM) 4 hr	Monatin (mM) 24 hr
wild type control	25%	0.90	2.80	12.40	12.80
Mutant 135	30%	0.50	8.80	12.40	12.40
Mutant 136	35%	3.80	7.80	11.60	12.80
Mutant M	15%	3.40	6.80	12.10	12.20
Mutant O	15%	5.20	8.60	10.90	9.80
Mutant BB	15%	3.40	6.20	10.50	12.60

The initial rate of monatin production observed with mutants Mutant 136, Mutant M, Mutant O, and Mutant BB was faster than the rate with the wild type control (SEQ ID NO:220). All the mutants showed improved monatin formation at 2 hours under the conditions tested. The lower than expected monatin titer at 1 hour for Mutant 135 was attributed to the inadvertent exposure to oxygen during the first hour. After 4 hours, the monatin titer was comparable between the mutants and the control under the conditions tested.

Example 35—Evaluation of the Impact of Temperature on Mutant DATs in BioReactors

This example describes data demonstrating the enzymatic activity of exemplary polypeptides disclosed herein under different temperature conditions. The wild type control (SEQ ID NO:220), Mutant 135 and Mutant M were produced in a fermentor at the 2.5 L scale as described in Example 15. At the end of fermentation, the cells were harvested by centrifugation at 5000-7000 x g for 10 minutes and frozen as a wet cell paste at -80°C.

To prepare cell free extract containing the wild type control, Mutant 135 and Mutant M D-aminotransferases, 50 g of wet cell paste was suspended in 150 mL of 50 mM potassium phosphate buffer (pH 7.8) containing 0.05 mM pyridoxal phosphate (PLP) and then disrupted using a Microfluidics homogenizer (Microfluidics, Newton, MA) (3 passes at 18,000 psi), maintaining the temperature of the suspension at less than 15°C. Cellular debris was removed by centrifugation (20,000 x g for 30 minutes).

The rate of formation of I3P from tryptophan was monitored at 340 nm for three minutes as described in Example 32. The concentration of the wild type control was determined to be 6.8 mg/mL, the concentration of Mutant 135 was determined to be 7.0 mg/mL and Mutant M was determined to be 5.6 mg/mL based on a standard curve generated with purified DAT wild type control. The DAT concentrations determined by I3P formation were used to dose the Infors to 0.2 mg/mL DAT. The aldolase was added as a cell free extract at 0.02 mg/mL aldolase. The reaction mix contained 10 mM potassium phosphate, 1 mM $MgCl_2$, 0.05 mM PLP, 200 mM sodium pyruvate and 130 mM D-tryptophan under a nitrogen headspace. Each of the DATs was evaluated for monatin production in a bioreactor at 35°C and at 25°C.

Samples were taken at 0.5, 1, 3, 4 and 24 hours and analyzed using the LC/MS/MS methodology described in Example 36. The results are shown in Table 60.

Table 60: Fermenters at 25° and 35°C

DAT polypeptide	Monatin (mM) 0.5 hr	Monatin (mM) 1 hr	Monatin (mM) 3 hr	Monatin (mM) 4 hr	Monatin (mM) 24 hr

25°C					
Wild type control (SEQ ID NO:220)	0.9	2.4	5.6	7.9	19.1
Mutant 135	1.6	4.4	10.9	12.1	18.6
Mutant M	2.1	4.5	9.4	12.4	17.4
35°C					
Wild type control (SEQ ID NO:220)	2.3	3.9	6.5	7.9	10.7
Mutant 135	4.1	6.1	9.8	11.5	14.9
Mutant M	4.1	6.3	9.9	11.3	14.9

As seen in Example 34, select mutant DATs yielded higher monatin titers at 35°C compared to the wild type control DAT (SEQ ID NO:220). The wild type control DAT had a slower initial rate of monatin production but a higher final titer at 25°C under the conditions tested. Both mutants Mutant 135 and Mutant M showed improved activity over the wild type control at 25°C and 35°C. Mutants Mutant 135 and Mutant M had both a higher initial rate of monatin production and a higher final titer at 35°C compared to the control under the conditions tested. The selected mutants were more stable than the wild type control at the higher temperatures. This indicates the advantages of GSSM and TMCA technologies in producing mutants with greater thermostability than the wild type control. One skilled in the arts could screen these GSSM or TMCA libraries for mutants with, for example, increased temperature tolerance.

Example 36—Detection of Monatin, MP, Tryptophan, Alanine, and HMG

This example describes the analytical methodology associated with the further characterization of exemplary D-aminotransferase (DAT) enzymes disclosed herein.

UPLC/UV Analysis of monatin and tryptophan

Analyses of mixtures for monatin and tryptophan derived from biochemical reactions were performed using a Waters Acquity UPLC instrument including a Waters Acquity Photo-Diode Array (PDA) absorbance monitor. UPLC separations were made using an Agilent XDB C8 1.8 μm 2.1 x 100 mm column (part # 928700-906) (Milford, MA) at 23°C. The UPLC mobile phase consisted of A) water containing 0.1% formic acid B) acetonitrile containing 0.1% formic acid.

The gradient elution was linear from 5% B to 40% B, 0-4 minutes, linear from 40% B, to 90% B, 4-4.2 minutes, isocratic from 90% B to 90% B, 4.2-5.2 minutes, linear from 90% B to

5% B, 5.2-5.3 minutes, with a 1.2 minute re-equilibration period between runs. The flow rate was 0.5 mL/min, and PDA absorbance was monitored at 280 nm.

Sample concentrations are calculated from a linear least squares calibration of peak area at 280 nm to known concentration, with a minimum coefficient of determination of 99.9%.

Derivatization of Monatin Intermediates (Indole-3-Pyruvic Acid (I3P), Hydroxymethyloxyglutaric Acid, Monatin Precursor, and Pyruvate) with O-(4-Nitrobenzyl)hydroxylamine hydrochloride (NBHA)

In the process of monatin production, various intermediate compounds are formed and utilized. These compounds include: Indole-3-Pyruvic Acid (I3P), Hydroxymethyloxyglutaric Acid, Monatin Precursor, and Pyruvate.. The ketone functional group on these compounds can be derivatized with O-(4-Nitrobenzyl)hydroxylamine hydrochloride (NBHA).

To 20 μL of sample or standard, 140 μL of NBHA (40 mg/mL in pyridine) was added in an amber vial. Samples were sonicated for 15 min in the presence of heat with occasional mixing. A 1:3 dilution in 35% Acetonitrile in water was performed.

UPLC/UV Analysis of Monatin Intermediates (Indole-3-Pyruvic Acid, Hydroxymethyloxyglutaric Acid, Monatin Precursor, and Pyruvate)

A Waters Acquity UPLC instrument including a Waters Acquity Photo-Diode Array (PDA) absorbance monitor (Waters, Milford, MA) was used for the analysis of the intermediate compounds. UPLC separations were made using a Waters Acquity HSS T3 1.8 mm x 150 mm column (Waters, Milford, MA) at 50°C. The UPLC mobile phase consisted of A) water containing 0.3% formic acid and 10 mM ammonium formate and B) 50/50 acetonitrile/methanol containing 0.3% formic acid and 10 mM ammonium formate.

The gradient elution was linear from 5% B to 40% B, 0-1.5 minutes, linear from 40% B, to 50% B, 1.5-4.5 minutes, linear from 50% B to 90% B, 4.5-7.5 minutes, linear from 90% B to 95% B, 7.5-10.5 minutes, with a 3 minute re-equilibration period between runs. The flow rate was 0.15 mL/min from 0-7.5 minutes, 0.18 mL/min from 7.5-10.5 minutes, 0.19 mL/min from 10.5-11 minutes, and 0.15 mL/min from 11-13.5 minutes. PDA absorbance was monitored at 270 nm.

Sample concentrations were calculated from a linear least squares calibration of peak area at 270 nm to known concentration, with a minimum coefficient of determination of 99.9%.

Chiral LC/MS/MS (MRM) Measurement of Monatin

Determination of the stereoisomer distribution of monatin in biochemical reactions was accomplished by derivatization with 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide 30 (FDAA), followed by reversed-phase LC/MS/MS MRM measurement.

Derivatization of Monatin with FDAA

100 μL of a 1% solution of FDAA in acetone was added to 50 μL of sample or standard. Twenty μL of 1.0 M sodium bicarbonate was added, and the mixture was incubated for 1 hour at 40°C with occasional mixing. The sample was removed and cooled, and neutralized with 20 μL of 2.0 M HCl (more HCl may be required to effect neutralization of a buffered biological mixture). Samples were analyzed by LC/MS/MS.

LC/MS/MS Multiple Reaction Monitoring for the Determination of the Stereoisomer Distribution of Monatin

Analyses were performed using the Waters/Micromass® liquid chromatography-tandem mass spectrometry (LC/MS/MS) instrument including a Waters 2795 liquid chromatograph with a Waters 996 Photo-Diode Array (PDA) absorbance monitor (Waters, Milford, MA) placed in series between the chromatograph and a Micromass® Quattro Ultima® triple quadrupole mass spectrometer. The LC separations capable of separating all four stereoisomers of monatin (specifically FDAA-monatin) were performed on a Phenomenex Luna® 2.0 x 250 mm (3 μm) C18 reversed phase chromatography column at 40°C. The LC mobile phase consisted of A) water containing 0.05% (mass/volume) ammonium acetate and B) acetonitrile. The elution was isocratic at 13% B, 0-2 minutes, linear from 13% B to 30% B, 2-15 minutes, linear from 30% B to 80% B, 15-16 minutes, isocratic at 80% B 16-21 minutes, and linear from 80% B to 13% B, 21-22 minutes, with a 8 minute re-equilibration period between runs. The flow rate was 0.23 mL/min, and PDA absorbance was monitored from 200 nm to 400 nm. All parameters of the ESI-MS were optimized and selected based on generation of deprotonated 20 molecular ions ([M - H]-) of FDAA-monatin, and production of characteristic fragment ions. The following instrumental parameters were used for LC/MS analysis of monatin in the negative ion ESI/MS mode: Capillary: 3.0 kV; Cone: 40 V; Hex 1: 15 V; Aperture: 0.1 V; Hex 2: 0.1 V; Source temperature: 120°C; Desolvation temperature: 350°C; Desolvation gas: 662 L/h; Cone gas: 42 L/h; Low mass resolution (Q1): 14.0; High mass resolution (Q1): 15.0; Ion energy: 0.5; Entrance: 0 V; Collision Energy: 20; Exit: 0 V; Low mass resolution (Q2): 15; High mass resolution (Q2): 14; Ion energy (Q2): 2.0; Multiplier: 650. Three FDAA-monatin-specific parent-to-daughter transitions were used to specifically detect FDAA-monatin in *in vitro* and *in*

vivo reactions. The transitions monitored for monatin were 542.97 to 267.94, 542.97 to 499.07, and 542.97 to 525.04. Identification of FDAA-monatin stereoisomers was based on chromatographic retention time as compared to purified monatin stereoisomers, and mass spectral data.

Liquid Chromatography-Post Column Derivatization with OPA, Fluorescence Detection of Amino Acids, including: Hydroxymethyl glutamate (HMG) and Alanine

Analyses of mixtures for HMG and alanine derived from biochemical reactions were performed using a Waters Alliance 2695 and a Waters 600 configured instrument with a Waters 2487 Dual Wavelengths Absorbance Detector and Waters 2475 Fluorescence Detector as a detection system (Waters, Milford, MA). HPLC separations were made using two Phenomenex Aqua C18 125A, 150 mm x 2.1 mm, 3 μ, Cat #00F-4311B0 columns in series as the analytical columns, and a Phenomenex Aqua C18 125A, 30 mm x 2.1 mm, 3 μ, Cat # 00A-4311B0 as an on-line solid phase extraction (SPE) column. Temperature for the two analytical columns was set at 55°C, and the on-line SPE column was at room temperature. The HPLC mobile phase consisted of A) 0.6% acetic acid with 1% methanol. The flow rate was (100% A) 0.2 mL/min from 0-3.5 minutes, 0.24 mL/min from 3.5-6.5 minutes, 0.26 mL/min from 6.5-10.4 minutes, and 0.2 mL/min from 10.4-11 minutes. UV-VIS absorbance detector was set to monitor at 336 nm wavelength. Fluorescence detector was set at 348 nm and 450 nm to monitor the excitation and emission wavelengths respectively.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of converting tryptophan to indole-3-pyruvate (or indole-3-pyruvate to tryptophan), comprising

combining tryptophan (or indole-3-pyruvate) with

a) one or more nucleic acid molecules chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 319, 321, 323, 325, 327, 329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, 357, 359, 361, 363, 365, 367, 369, 371, 373, 375, 377, 379, 381, 383, 385, 387, 389, 391, 393, 395, 397, 399, 401, 403, 405, 407, 409, 411, 413, 415, 417, 419, 421, 423, 425, 427, 429, 431, 433, 435, 437, 439, 441, 443, 445, 447, 449, 451, 453, 455, 457, 459, 461, 463, 465, 467, 469, 471, 473, 475, 477, 479, 481, 483, 485, 487, 489, 491, 493, 495, 497, 499, 501, 503, 505, 507, 509, 511, 513, 515, 517, 519, 521, 523, 525, 527, 529, 531, 533, 535, 537, 539, 541, 543, 545, 547, 549, 551, 553, 555, 557, 559, 561, 563, 565, 567, 569, 571, 573, 575, 577, 579, 581, 583, 585, 587 589, 591, 593, 595, 597, 599, 601, 603, 605, 607, 609, 611, 613, 615, 617, 619, 621, 623, 625, 627, 629, 631, 633, 635, 637, 639, 641, 643, 645, 647, 649, 651, 653, 655, 657, 659, 661, 663, 665, 667, 669, 671, 673, 675, 677, 679, 681, 683, 685, 687, 689, 691, 693, 695, 697, 699, 701, 703, 705, 707, 709, 711, 713, 715, 716, 719, 721, 723, 725, 727, 729, 731, 733, 735, 737, 739, 741, 743, 745, 747, 749, 751, 753, 755, 757, 759, 761, 763, 765, 767, 769, 771, 773, 775, 777, 779, 781, 783, 785, 787, 789, 791, 793, 795, 797, 799, 801, 803, 805, 807, 809, 811, 813, 815, 817, 819, 821, 823, 825, 827, 829, 831, 833, 835, 837, 839, 841, 843, 845, 847, 849, 851, 853, 855, 857, 859, 861, 863, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 961, 963, 965, 967, 969, 971, 973, and 975, wherein said one or more nucleic acid molecules encode polypeptides having aminotransferase (AT) or oxidoreductase activity;

b) a variant of a), wherein said variant encodes a polypeptide having AT or oxidoreductase activity;

c) a fragment of a) or b), wherein said fragment encodes a polypeptide having AT or oxidoreductase activity;

d) one or more polypeptides chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572, 574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 962, 964, 966, 968, 970, 972, 974, 976, 1069, 1070, 1071, 1072 and 1073, wherein said one or more polypeptides has AT or oxidoreductase activity;

e) a variant of d), wherein said variant has AT or oxidoreductase activity; or

f) a fragment of d) or e), wherein said fragment has AT or oxidoreductase activity.

2. A method of converting MP to monatin (or monatin to MP), comprising combining MP (or monatin) with

a) one or more nucleic acid molecules chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 251, 253, 255, 257, 259, 261, 263, 265, 267, 269, 271, 273, 275, 277, 279, 281, 283, 285, 287, 289, 291, 293, 295, 297, 299, 301, 303, 305, 307, 309, 311, 313, 315, 317, 319, 321, 323, 325, 327, 329, 331, 333, 335, 337, 339, 341, 343, 345, 347, 349, 351, 353, 355, 357, 359, 361, 363, 365, 367, 369, 371, 373, 375, 377, 379, 381, 383, 385, 387, 389, 391, 393, 395, 397, 399, 401, 403, 405, 407, 409, 411, 413, 415, 417, 419, 421, 423, 425, 427, 429, 431, 433, 435, 437, 439, 441, 443, 445, 447, 449, 451, 453, 455, 457, 459, 461, 463, 465, 467, 469, 471, 473, 475, 477, 479, 481, 483, 485, 487, 489, 491, 493, 495, 497, 499, 501, 503, 505, 507, 509, 511, 513, 515, 517, 519, 521, 523, 525, 527, 529, 531, 533, 535, 537, 539, 541, 543, 545, 547, 549, 551, 553, 555, 557, 559, 561, 563, 565, 567, 569, 571, 573, 575, 577, 579, 581, 583, 585, 587 589, 591, 593, 595, 597, 599, 601, 603, 605, 607, 609, 611, 613, 615, 617, 619, 621, 623, 625, 627, 629, 631, 633, 635, 637, 639, 641, 643, 645, 647, 649, 651, 653, 655, 657, 659, 661, 663, 665, 667, 669, 671, 673, 675, 677, 679, 681, 683, 685, 687, 689, 691, 693, 695, 697, 699, 701, 703, 705, 707, 709, 711, 713, 715, 716, 719, 721, 723, 725, 727, 729, 731, 733, 735, 737, 739, 741, 743, 745, 747, 749, 751, 753, 755, 757, 759, 761, 763, 765, 767, 769, 771, 773, 775, 777, 779, 781, 783, 785, 787, 789, 791, 793, 795, 797, 799, 801, 803, 805, 807, 809, 811, 813, 815, 817, 819, 821, 823, 825, 827, 829, 831, 833, 835, 837, 839, 841, 843, 845, 847, 849, 851, 853, 855, 857, 859, 861, 863, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 961, 963, 965, 967, 969, 971, 973, and 975, wherein said one or more nucleic acid molecules encode polypeptides having aminotransferase (AT) or oxidoreductase activity;

b) a variant of a), wherein said variant encodes a polypeptide having AT or oxidoreductase activity;

c) a fragment of a) or b), wherein said fragment encodes a polypeptide having AT or oxidoreductase activity;

d) one or more polypeptides chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 252, 254, 256, 258, 260, 262, 264, 266, 268, 270, 272, 274, 276, 278, 280, 282, 284, 286, 288, 290, 292, 294, 296, 298, 300, 302, 304, 306, 308, 310, 312, 314, 316, 318, 320, 322, 324, 326, 328, 330, 332, 334, 336, 338, 340, 342, 344, 346, 348, 350, 352, 354, 356, 358, 360, 362, 364, 366, 368, 370, 372, 374, 376, 378, 380, 382, 384, 386, 388, 390, 392, 394, 396, 398, 400, 402, 404, 406, 408, 410, 412, 414, 416, 418, 420, 422, 424, 426, 428, 430, 432, 434, 436, 438, 440, 442, 444, 446, 448, 450, 452, 454, 456, 458, 460, 462, 464, 466, 468, 470, 472, 474, 476, 478, 480, 482, 484, 486, 488, 490, 492, 494, 496, 498, 500, 502, 504, 506, 508, 510, 512, 514, 516, 518, 520, 522, 524, 526, 528, 530, 532, 534, 536, 538, 540, 542, 544, 546, 548, 550, 552, 554, 556, 558, 560, 562, 564, 566, 568, 570, 572, 574, 576, 578, 580, 582, 584, 586, 588, 590, 592, 594, 596, 598, 600, 602, 604, 606, 608, 610, 612, 614, 616, 618, 620, 622, 624, 626, 628, 630, 632, 634, 636, 638, 640, 642, 644, 646, 648, 650, 652, 654, 656, 658, 660, 662, 664, 666, 668, 670, 672, 674, 676, 678, 680, 682, 684, 686, 688, 690, 692, 694, 696, 698, 700, 702, 704, 706, 708, 710, 712, 714, 716, 718, 720, 722, 724, 726, 728, 730, 732, 734, 736, 738, 740, 742, 744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780, 782, 784, 786, 788, 790, 792, 794, 796, 798, 800, 802, 804, 806, 808, 810, 812, 814, 816, 818, 820, 822, 824, 826, 828, 830, 832, 834, 836, 838, 840, 842, 844, 846, 848, 850, 852, 854, 856, 858, 860, 862, 864, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 962, 964, 966, 968, 970, 972, 974, 976, 1069, 1070, 1071, 1072 and 1073, wherein said one or more polypeptides has AT or oxidoreductase activity;

e) a variant of d), wherein said variant has AT or oxidoreductase activity; or

f) a fragment of d) or e), wherein said fragment has AT or oxidoreductase activity.

3. The method of claim 1 or 2, wherein said one or more nucleic acid molecules are chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 969, 971, 973, and 975.

4. The method of claim 1 or 2, wherein said one or more polypeptides are chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, and 976.

5. The method of claim 1 or 2, wherein the nucleic acid molecule has a sequence selected from the group consisting of SEQ ID NOs:945, 891, 893, 219, 175, 1063, 1065, and 1067.

6. The method of claim 1 or 2, wherein the polypeptide has a sequence selected from the group consisting of SEQ ID NOs:946, 892, 894, 220, 176, 1064, 1066, and 1068.

7. The method of claim 1 or 2, wherein the polypeptide has a sequence that corresponds to the consensus sequence shown in SEQ ID NO:1069 or 1070.

8. The method of claim 1 or 2, wherein the polypeptide has a sequence that corresponds to the consensus sequence shown in SEQ ID NO:1071, 1072, and 1073.

9. The method of claim 1 or 2, wherein said variant is a nucleic acid molecule that has at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 969, 971, 973, and 975.

10. The method of claim 1 or 2, wherein said variant is a polypeptide that has at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, and 976.

11. The method of claim 1 or 2, wherein said variant is a polypeptide that has at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:220.

12. The method of claim 1 or 2, wherein said variant is a polypeptide that has at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:870.

13. The method of claim 1 or 2, wherein said variant is a polypeptide that has at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, or at least 99% sequence identity to SEQ ID NO:894.

14. The method of claim 1 or 2, wherein said variant is a mutant.

15. The method of claim 14, wherein the mutant has a mutation at the residue that aligns with residue 243 of DAT 4978.

16. The method of claim 15, wherein the mutant is selected from the group consisting of SEQ ID NO:870 T242N, SEQ ID NO:220 G240N, and SEQ ID NO:220 T241N.

17. The method of claim 1 or 2, wherein the variant is a nucleic acid molecule that has been codon optimized.

18. The method of claim 1 or 2, wherein the nucleic acid molecule is contained within an expression vector.

19. The method of claim 18, wherein the nucleic acid molecule is overexpressed.

20. The method of claim 1 or 2, wherein the aminotransferase or oxidoreductase polypeptide is immobilized on a solid support.

21. The method of claim 1 or 2, wherein the variant polypeptide is a chimeric polypeptide.

22. The method of claim 1 or 2, wherein the tryptophan or the MP is a substituted tryptophan or a substituted MP.

23. A method of converting tryptophan to indole-3-pyruvate (or indole-3-pyruvate to tryptophan), comprising

combining tryptophan (or indole-3-pyruvate) with

a) one or more nucleic acid molecules chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 969, 971, 973, and 975, wherein said one or more nucleic acid molecules encode polypeptides having D-aminotransferase (DAT) activity;

b) a variant of a), wherein said variant encodes a polypeptide having DAT activity;

c) a fragment of a) or b), wherein said fragment encodes a polypeptide having DAT activity;

d) one or more polypeptides chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, 976, 1069, 1070, 1071, 1072 and 1073, wherein said one or more polypeptides has DAT activity;

e) a variant of d), wherein said variant has DAT activity; or

f) a fragment of d) or e), wherein said fragment has DAT activity.

24. A method of converting MP to monatin (or monatin to MP), comprising combining MP (or monatin) with

a) one or more nucleic acid molecules chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47,

49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 969, 971, 973, and 975, wherein said one or more nucleic acid molecules encode polypeptides having D-aminotransferase (DAT) activity;

b) a variant of a), wherein said variant encodes a polypeptide having DAT activity;

c) a fragment of a) or b), wherein said fragment encodes a polypeptide having DAT activity;

d) one or more polypeptides chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, 976, 1069, 1070, 1071, 1072 and 1073, wherein said one or more polypeptides has DAT activity;

e) a variant of d), wherein said variant has DAT activity; or

f) a fragment of d) or e), wherein said fragment has DAT activity.

25. The method of claim 23 or 24, wherein the nucleic acid molecule or polypeptide has a sequence selected from the group consisting of SEQ ID NO:945, 891, 893, 219, 175, 1063, 1065, and 1067.

26. The method of claim 23 or 24, wherein the polypeptide has a sequence that corresponds to a consensus sequence shown in SEQ ID NO:1069, 1070, 1071, 1072 or 1073.

27. The method of claim 23 or 24, wherein the tryptophan or the MP is a substituted tryptophan or a substituted MP.

28. A method of making monatin, comprising

contacting tryptophan, under conditions in which monatin is produced, with a C3 carbon source and

a) one or more nucleic acid molecules chosen from the group consisting of SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81, 83, 85, 87, 89, 91, 93, 95, 97, 99, 101, 103, 105, 107, 109, 111, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133, 135, 137, 139, 141, 143, 145, 147, 149, 151, 153, 155, 157, 159, 161, 163, 165, 167, 169, 171, 173, 175, 177, 179, 181, 183, 185, 187, 189, 191, 193, 195, 197, 199, 201, 203, 205, 207, 209, 211, 213, 215, 217, 219, 221, 223, 225, 227, 229, 231, 233, 235, 237, 239, 241, 243, 245, 247, 249, 865, 867, 869, 871, 873, 875, 877, 879, 881, 883, 885, 887, 889, 891, 893, 895, 897, 899, 901, 903, 905, 907, 909, 911, 913, 915, 917, 919, 921, 923, 925, 927, 929, 931, 933, 935, 937, 939, 941, 943, 945, 947, 949, 951, 953, 955, 957, 959, 969, 971, 973, and 975, wherein said one or more nucleic acid molecules encode polypeptides having D-aminotransferase (DAT) activity;

b) a variant of a), wherein said variant encodes a polypeptide having DAT activity;

c) a fragment of a) or b), wherein said fragment encodes a polypeptide having DAT activity;

d) one or more polypeptides chosen from the group consisting of SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82, 84, 86, 88, 90, 92, 94, 96, 98, 100, 102, 104, 106, 108, 110, 112, 114, 116, 118, 120, 122, 124, 126, 128, 130, 132, 134, 136, 138, 140, 142, 144, 146, 148, 150, 152, 154, 156, 158, 160, 162, 164, 166, 168, 170, 172, 174, 176, 178, 180, 182, 184, 186, 188, 190, 192, 194, 196, 198, 200, 202, 204, 206, 208, 210, 212, 214, 216, 218, 220, 220 G240N, 220 T241N, SEQ ID NO:220 having one or more of the mutations shown in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248, 250, 866, 868, 870, 870 T242N, 872, 874, 876, 878, 880, 882, 884, 886, 888, 890, 892, 894, 896, 898, 900, 902, 904, 906, 908, 910, 912, 914, 916, 918, 920, 922, 924, 926, 928, 930, 932, 934, 936, 938, 940, 942, 944, 946, 948, 950, 952, 954, 956, 958, 960, 970, 972, 974, 976, 1069, 1070, 1071, 1072 and 1073, wherein said one or more polypeptides has DAT activity;

e) a variant of d), wherein said variant has DAT activity; or

f) a fragment of d) or e), wherein said fragment has DAT activity.

29. The method of claim 28, wherein the nucleic acid molecule or polypeptide is chosen from the group consisting of SEQ ID NO: 945, 891, 893, 219, 175, 1063, 1065, and 1067.

30. The method of claim 28, wherein said C3 carbon source is selected from the group consisting of pyruvate, oxaloacetate, and serine.

31. The method of claim 28, further comprising a synthase / lyase (EC 4.1.2.- or 4.1.3.-) polypeptide.

32. The method of claim 31, wherein the synthase / lyase (EC 4.1.2.- or 4.1.3.-) polypeptide is an aldolase.

33. The method of claim 32, wherein the aldolase is selected from the group consisting of a KHG aldolase (EC 4.1.3.16) or a HMG aldolase (EC 4.1.3.16).

34. The method of claim 28, wherein the monatin produced is R,R monatin.

35. The method of claim 28, wherein the monatin produced is S,R monatin.

36. The method of claim 28, wherein the tryptophan is a substituted tryptophan.

37. The method of claim 36, wherein the substituted tryptophan is 6-chloro-D-tryptophan.

Figure 1

```
SEQ ID NO:894    MDALGYYNGKWGPLDEMTVPMNDRGGFFGDGVYDATIAANGVTFALDEHTDRFLNSAKLLEIEIGFTKEELKKTFFEM--
SEQ ID NO:1066   MENLGYYNGKFGLLEEMTVPMLDRVGYFGDGVYDATYSRNHKTFALEEHTDRFYNSAGLLGIKIPYSKEQVKEILKEM--
SEQ ID NO:1064   MKDLGYYNGEYDLIENMKIPMNDRVGYFGDGVYDATYSRNHNTFALDEHTDRFYNSAELLRIKIPYTKKEMKELLKDM--
SEQ ID NO:1068   MKQVGYYNGTIADINELKVPATDRALYFGDGCYDATTFKNNVAFALEDHLDRFYNSCRLLEIDFPLNRDELKEKLYAVID

SEQ ID NO:894    HSKVDKGVYMVYWQATRGTGRRSHVFPAG--P--S-NLWTMIKPNHVDDLYRKIKLITMEDTRFLHGNIKTLNLIPNVLA
SEQ ID NO:1066   VLKVDSGEQFVYWQITRGTGMRNHAFPGDEVP--S-NLWTMLKPLNIKDMSQKLKLITLEDTRFLHGNIKTLNLLPSVLA
SEQ ID NO:1064   VKKVDSGEQFVYWQVTRGTGMRNHAFLSE--DKVA-NIWTVLKPLKVKDMSKKLKLITLEDTRFLHGNIKTLNLLPSVLA
SEQ ID NO:1068   ANEVDTGI--LYWQTSRGSGLRNHIFPED--S--QPNLLIFTAEYGLVPFDTEYKLISREDTRFLHGNIKTLNLLPNVLA

SEQ ID NO:894    SQRALEAGGHEAVFHRGETVTEGAHSNVHLIKNGRFITHQADNLILRGLARSHLLQAGIRLNIPFDEREFLLSELFDADE
SEQ ID NO:1066   SQKTEEAGGQEAVFHRGDRVTEGAHSNVSLIKDGILKTAPTDNLILPGLARAHLIKMGKSFNIPVDETAFTLKELMEADE
SEQ ID NO:1064   AQKTEEAGGQEAVFHRGDRVTEGAHSNVSLIKDEILKTAPTDNLILPGLARAHLIKMGKKFEIPVDETPFTLKELINADE
SEQ ID NO:1068   SQKANESHGQEVVFHRGDRVTEGAHSNILLLKDGVLCSPPRDNLILPGITLKHLLQLAKENNIPTSEAPFTMDDLRNADE

SEQ ID NO:894    ILVSSSGTLGLSANTIDGKNVGGKAPELLKKIQGEVLREFIEATGYTPEWSTV*
SEQ ID NO:1066   VIVTSSGQFCMATSEIDGIPVGGKAPELVKKLQDALNEFLEETKTE-------
SEQ ID NO:1064   VIVTSSGQFCMTACEIDGRPVGGKAPDIIKKLQTALNEFLEETN---------
SEQ ID NO:1068   VIVSSSACLGIRAVELDGQPVGGKDGKTLKILQDAYAKKYNAETVSR-------
```

Figure 2

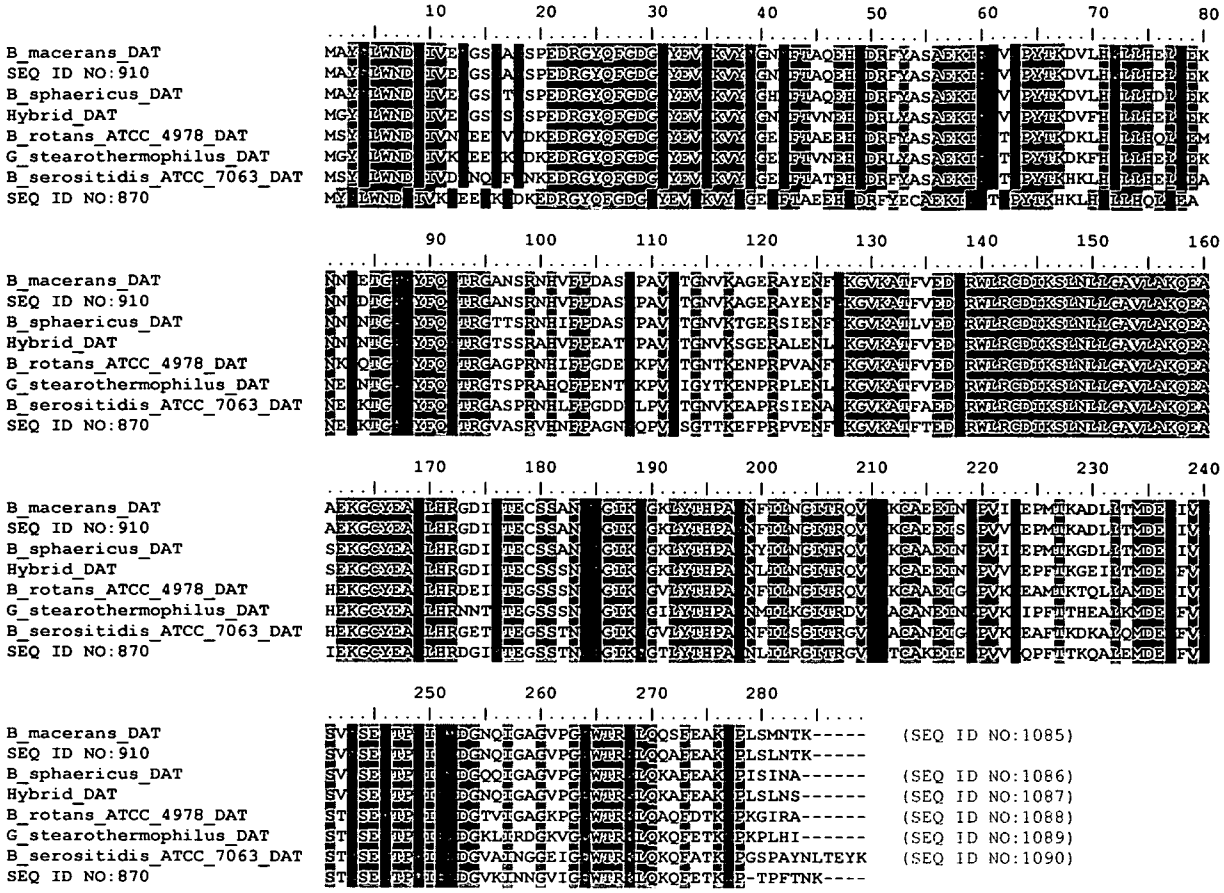

B_macerans_DAT
SEQ ID NO:910
B_sphaericus_DAT
Hybrid_DAT
B_rotans_ATCC_4978_DAT
G_stearothermophilus_DAT
B_serositidis_ATCC_7063_DAT
SEQ ID NO:870
(SEQ ID NO:1085)
(SEQ ID NO:1086)
(SEQ ID NO:1087)
(SEQ ID NO:1088)
(SEQ ID NO:1089)
(SEQ ID NO:1090)

Figure 3

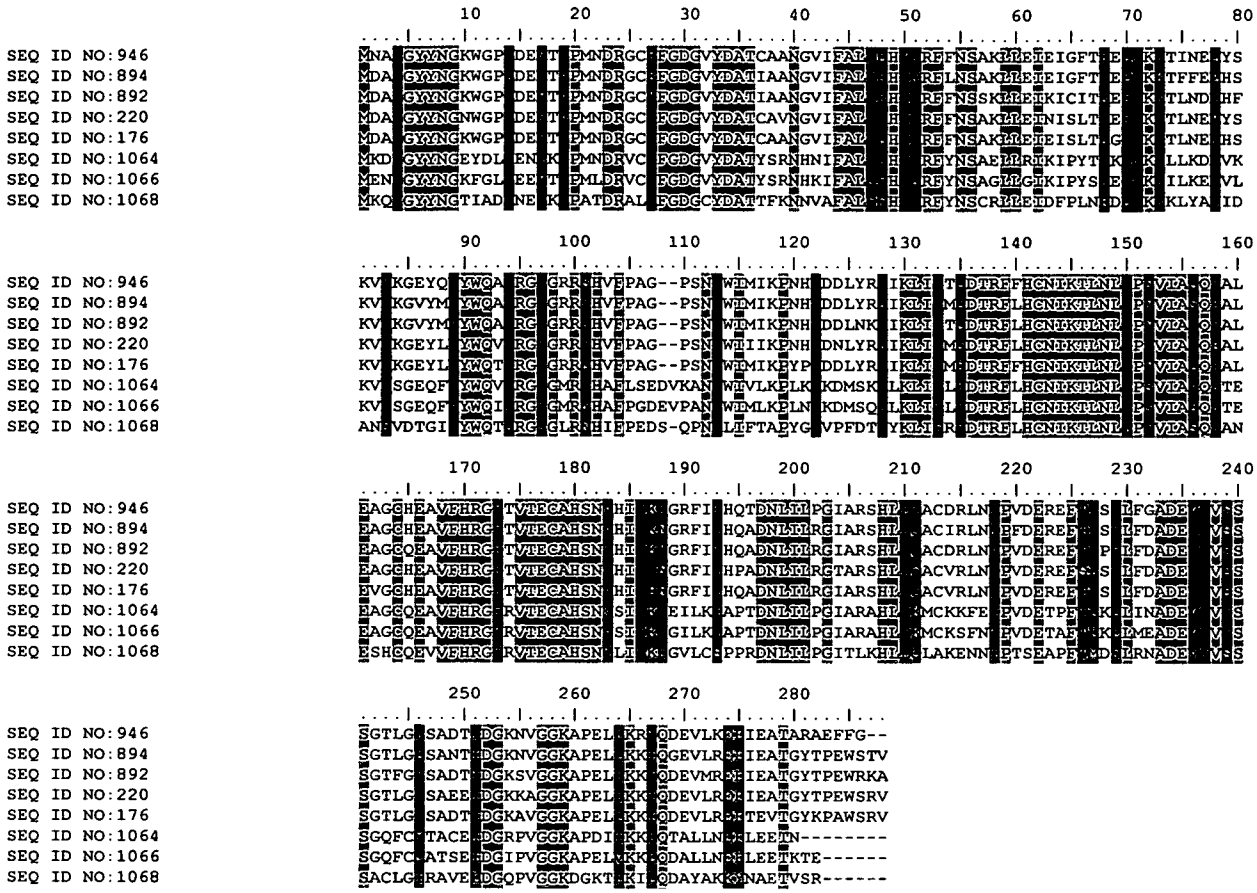
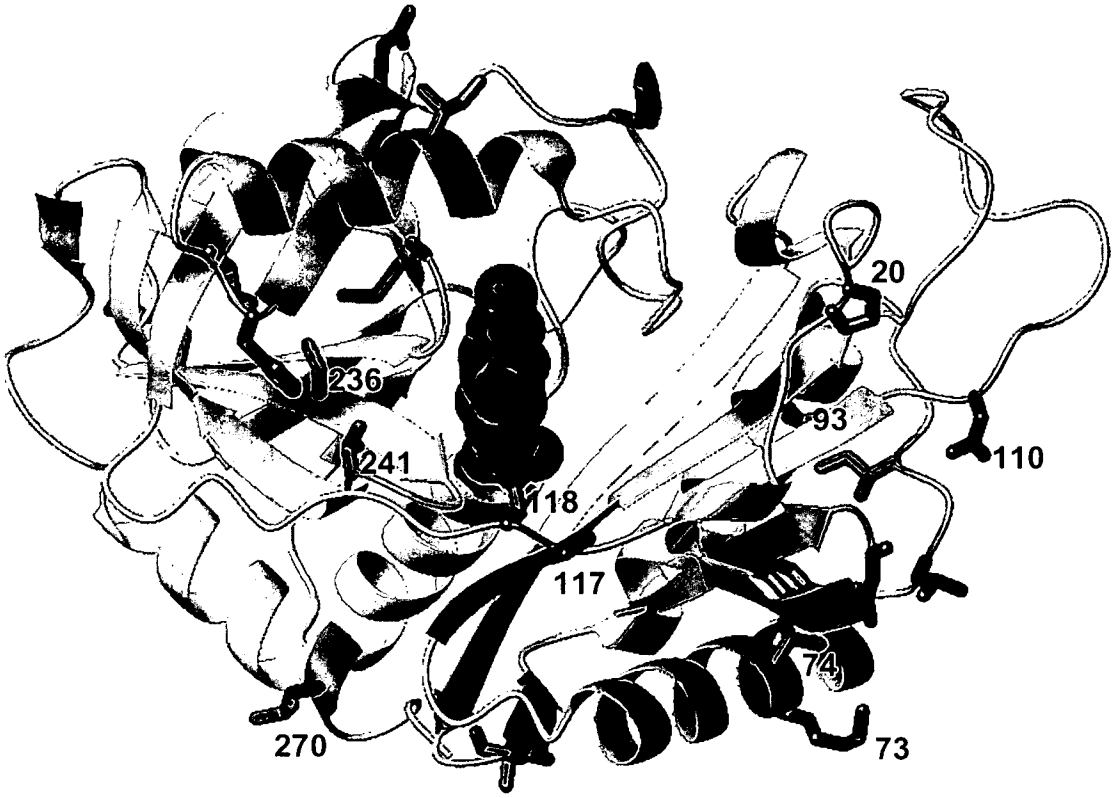

SEQ ID NO:946
SEQ ID NO:894
SEQ ID NO:892
SEQ ID NO:220
SEQ ID NO:176
SEQ ID NO:1064
SEQ ID NO:1066
SEQ ID NO:1068

Figure 4



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/14137

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 21/06; C12N 9/10 (2009.01)
USPC - 435/69.1; 435/193
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC - 435/69.1, 193

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/6, 108; 536/23.2 (text search, see terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST(PGPB,USPT,EPAB,JPAB); Google/Scholar; PubMed (text search, see terms below)
Search Terms: D-aminotransferase, aminotransferase, transaminase, indol-3-pyruvate, monatin, tryptophan, variant, mutant, chimeric, codon optimized, monatin precursor, MP, 6-chloro-D-tryptophan, 6-chloro-tryptophan, chloro, halogenated.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2006/0252135 A1 (BRAZEAU et al.) 9 November 2006 (09.11.2006) Fig 3; para [0009], [0064], [0070], [0075], [0097], [0207], [0212], [0228], [0387], [0397]	1-2, 9-10, 14-15, 17-24, 27-28, 30-37
A		3-4
Y	WO 2004/085624 A2 (ZHAO et al.) 7 October 2004 (07.10.2004) SEQ ID NO:5, nt 76-954; SEQ ID NO:6, aa 26-317; pg 16, ln 4-14	1-2, 9-10, 14-15, 17-24 27-28, 30-37
A		3-4
Y	US 2005/0106305 A1 (ABRAHAM et al.) 19 May 2005 (19.05.2005) para [0220]	22, 27, 36-37
Y	Moriya et al. A facile synthesis of 6-chloro-D-tryptophan. Bulletin of the Chemical Society of Japan, 1975, Vol 48, pp 2217-2218, Abstract	22, 27, 36-37

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 May 2009 (25.05.2009)	04 JUN 2009
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/14137

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-37, limited to nucleic acid sequence SEQ NO:1 and polypeptide SEQ NO:2, namely claims 1-4, 9-10, 14-15, 17-24, 27-28 and 30-37

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/14137

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Inventions 1-490: Nucleic acids having SEQ ID NOs:1, 3, 5, 7, 9,11,13,15,17,19,21,23,25,27,29,31,33,35,37,39,41, 43, 45,
47,49,51,53,55,57,59,61,63,65,67,69,71,73,75,77, 79, 81,83,85,87,89,91,93,95,97,99,
101,103,105,107,109, III, 113, 115, 117, 119, 121, 123, 125, 127, 129, 131, 133,135,137,
139,141,143,145,147,149,151,153,155,157,159,161, 163, 165, 167, 169, 171, 173, 175,
177,179,181,183,185,187,189,191,193,195,197,199, 201, 203, 205, 207, 209, 211, 213,
215,217,219,221,223,225,227,229,231,233,235,237,239,241,243,245,247,249,251,
253,255,257,259,261,263,265,267,269,271,273,275,277,279,281,283,285,287,289,
291,293,295,297,299,301,303,305,307,309,311,313, 315, 317, 319,321,323, 325, 327,
329,331,333,335,337,339,341,343,345,347,349,351,353,355,357,359,361,363,365,
367,369,371,373,375,377,379,381,383,385,387,389,391,393,395,397,399,401,403,
405,407,409,411,413,415,417,419,421,423,425,427,429,431,433,435,437,439,441,
443,445,447,449,451,453,455,457,459,461,463,465,467,469,471,473,475,477,479,
481,483,485,487,489,491,493,495,497,499,501,503,505,507,509,511,513,515,517,
519,521,523,525,527, 529,531,533,535, 537,539,541,543,545,547,549, 551, 553,555,
557,559,561,563, 565, 567,569,571,573,575,577,579,581,583,585,587 589,591, 593,
595,597,599,601,603,605,607,609,611,613,615,617,619,621,623,625,627,629,631,
633,635,637,639,641,643,645,647,649,651,653,655,657,659,661,663,665,667,669,
671,673,675,677,679, 681,683,685,687,689,691,693, 695,697,699, 701, 703, 705, 707,
709,711,713,715,716,719,721,723,725,727,729,731, 733, 735, 737, 739, 741, 743, 745,
747,749,751,753,755,757,759,761,763,765,767,769, 771, 773, 775, 777, 779, 781,783,
785,787,789,791,793,795,797,799,801, 803,805,807, 809,811,813,815,817,819,821,
823,825,827,829, 831,833,835,837,839, 841,843,845,847,849,851,853, 855, 857,859,
861,863,865,867,869,871,873,875,877,879,881, 883, 885,887, 889, 891, 893, 895, 897,
899,901,903,905,907,909,911,913,915,917,919,921,923,925,927,929,931,933,935,
937,939,941,943,945,947,949,951,953,955,957,959,961,963,965,967,969,971,973,
and 975 and polypeptide sequences having SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20,22, 24, 26, 28, 30, 32, 34, 36, 38; 40, 42, 44,
46, 48, 50, 52,54,56,58,60,62,64,66,68,70,72,74,76, 78,80, 82,84,86,88,90,92,94,96,98,100,102,
104,106,108,110,112,114,116,118,120,122,124,126, 128, 130, 132, 134, 136,138,140,
142,144,146,148,150,152,154,156,158,160,162,164, 166,168,170,172,174,176,178,
180,182,184,186,188,190,192,194,196,198,200,202, 204, 206, 208, 210, 212, 214,216,
218, 220, 220 G240N, 220 T241 N, SEQ ID NO:220 having one or more of the mutations shown
in Table 43 or Table 52, 222, 224, 226, 228, 230, 232, 234, 236, 238, 240, 242, 244, 246, 248,
250,252,254,256,258,260,262,264,266,268,270,272,274,276,278,280,282,284,286,
288,290,292,294,296,298,300,302,304,306,308,310,312,314,316,318,320,322,324,
326,328,330,332,334,336,338,340,342,344,346,348, 350, 352,354, 356,358,360, 362,
364,366,368,370,372,374,376,378,380,382,384,386,388,390,392,394,396,398,400,
402,404,406,408,410,412,414,416,418,420,422,424,426,428,430,432,434,436,438,
440,442,444,446,448,450,452,454,456,458,460,462,464,466,468,470,472,474,476,
478,480,482,484,486,488,490,492,494,496,498,500,502, 504,506,508,510,512, 514,
516,518,520,522,524,526,528,530,532,534,536,538, 540, 542, 544, 546, 548,550, 552,
554,556,558,560,562,564, 566,568,570,572,574,576,578, 580,582,584,586,588,590,
592, 594,596,598,600,602,604,606,608,610,612,614,616,618,620,622,624,626,628,
630,632,634,636,638,640,642,644,646,648,650,652,654,656,658,660,662,664,666,
668,670,672,674,676,678,680,682,684,686,688,690,692,694,696,698,700,702,704,
706,708,710,712,714,716,718,720,722,724,726,728, 730, 732, 734, 736,738,740,742,
744, 746, 748, 750, 752, 754, 756, 758, 760, 762, 764, 766, 768, 770, 772, 774, 776, 778, 780,
782,784,786,788,790,792,794,796, 798,800,802,804,806, 808,810, 812,814, 816, 818,
820,822,824,826,828,830,832,834, 836,838,840,842,844, 846,848,850,852, 854,856,
858,860,862,864,866,868, 870, 870T242N, 872,874,876,878,880,882, 884,886,888,890,
892,894,896,898,900,902,904, 906,908,910,912,914,916, 918,920,922,924, 926, 928,
930,932,934,936,938,940,942,944,946,948,950,952,954,956,958,960,962,964,966,
968,970,972,974,976,1069,1070,1071,1072 and 1073.

The inventions listed as Inventions 1-490 do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
The nucleic acid having the SEQ ID NOs: identified above and the polypeptide sequences having the SEQ ID NOs: identified do not share any significant structural element and cannot be considered as having the same or corresponding technical feature.

The inventions listed as Inventions 1-490 do not relate to a single general inventive concept under PCT Rule 13.1 because under PCT Rule 13.2 they lack the same or corresponding special technical feature. According to PCT Rule 13.2, unity of invention exists only when the same or corresponding technical feature is shared by all claimed inventions. Note: with combinations and multiple sequences, the number of inventions is much greater than 490.

The different physical characteristics of the sequences represent distinct traits that are measured using different techniques and compared using different criteria. The different polypeptide sequences represented by the amino acid content of the species (and their related polynucleotide sequences) are different structures that are not common to one another but are different because they are composed of unique amino acid (nucleic acid) sequences.

In this case the first named invention and first named species that will be searched without additional fees is Group I represented by 1-37, limited to nucleic acid sequence SEQ NO:1 and polypeptide SEQ NO:2, namely claims 1-4, 9-10, 14-15, 17-24, 27-28 and 30-37.

In order for more than one species (invention) to be examined, the appropriate additional examination fees must be paid and the desired species clearly identified.